

A DNA-Encoded Macrocycle Library that Resembles Natural Macrocycles

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Herein we perform a seven-step chemical synthesis of a DNA-encoded macrocycle library (DEML) on DNA. Inspired by polyketide and mixed peptide-polyketide natural products, the library was designed to incorporate rich backbone diversity. Achieving this diversity, however, comes at the cost of custom synthesis of bifunctional building block libraries. Our work outlines the importance of careful retrosynthetic design in DNA-encoded libraries, while revealing areas where new DNA synthetic methods are needed.

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A DNA-encoded macrocycle library that resembles natural macrocycles

Cedric J. Stress,^[a] Basilius Sauter, Lukas A. Schneider, Timothy Sharpe and Dennis Gillingham^{*[a]}

Abstract: Herein we perform a seven-step chemical synthesis of a DNA-encoded macrocycle library (DEML) on DNA. Inspired by polyketide and mixed peptide-polyketide natural products, the library was designed to incorporate rich backbone diversity. Achieving this diversity, however, comes at the cost of custom synthesis of bifunctional building block libraries. Our work outlines the importance of careful retrosynthetic design in DNA-encoded libraries, while revealing areas where new DNA synthetic methods are needed.

Nearly all approved macrocyclic drugs are natural products or close derivatives.^[1] Developing novel man-made macrocycle drugs is challenging and unpredictable because they violate all or some of Lipinski's rules for oral bioavailability.^[2] A second problem is that non-peptidic macrocycles are not amenable to chemical optimization the way that small molecule drugs are. Macrocyclic 'hits' are often highly optimized and synthetically cumbersome (because they are large and derived from natural products), with little opportunity for chemical tuning. Macrocyclic libraries to date have largely focused on side-chain diversity, but recent work suggests that the backbone is intimately involved in binding of macrocycles to their targets.^[3] In fact natural macrocycles as a class are characterized by highly diverse backbones rather than side-chains.^[1, 4] Some great strides have been made in understanding the properties of macrocycles that improve their prospects as drugs, despite their seemingly unfavorable physicochemical properties. The Whitty^[3, 5] and Kihlberg^[6] groups have independently examined large collections of macrocycles to try and identify a set of actionable rules for predicting oral bioavailability beyond the rule-of-five. A key feature seems to be a macrocycle's ability to minimize polar surface area when exposed to non-polar solvents. This 'chameleonic' property is likely the source of many macrocycle's ability to maintain cell permeability despite their large size. But these datasets are based on approved drugs from Nature's bounty of macrocycles. A bigger collection of macrocycles to screen and study would allow us to determine whether they are truly unusual in terms of cell permeability and protein binding; or whether our current thinking is biased by the compound set we have been given by natural evolution. In this vein, a number of exciting methods, both biological^[7] and chemical,^[8] have recently been developed to create large collections of encoded macrocycles. In particular, the Liu lab has created a backbone diverse DNA-encoded macrocycle library (DEML),^[8a-c] while the

Neri lab^[8d] has reported a highly side-chain diverse DEML built on a constant peptide scaffold (see Fig. 1a & B). Missing from the current set is a library that includes more hydrophobic components in the backbone. Given this background, we focused on the construction of an encoded natural product-like DEML with diverse ring scaffolds. Scaffold diversity, however, comes at a high synthetic cost since commercial libraries of bifunctional hydrocarbon precursors are not available. Here we report the synthesis and preliminary evaluation of a DEML where the backbone is inspired by the polyketide and mixed peptide-polyketide macrocyclic natural products. The DEML contains over two thousand distinct scaffolds, which, along with its side-chain diversity, gives a total library size of over a million members.

Modern drug discovery typically begins with screening diverse compound libraries to find 'hits' against a given target, which are then further optimized into lead structures for *in vivo* testing. However, high throughput screening (HTS) of compound collections has a large infrastructure cost and throughput scales increase linearly with the size of the compound collection since each compound must be spatially separated. Technologies that allow screening of pooled compound libraries offer an alternative, but comes with the problem of accurate hit deconvolution after selection.^[9] Biological and biochemical selection techniques offer an elegant solution because the genotype (DNA) and the phenotype (aptamer, protein) are physically connected. Thus, after a functional or binding selection, the structure of best performers can be determined by standard DNA sequencing. The advent of next generation sequencing has further revolutionized the field because now information on entire pools of selected molecules can be gleaned, offering a more nuanced interpretation of selection datasets. Selection approaches were restricted to natural systems until Brenner and Lerner, inspired by the success of monoclonal antibody development with phage display libraries,^[9] proposed in the early nineties that sequential synthesis and encoding on a chimeric bead might allow selection of wholly artificial molecules.^[10] It took several years for this concept to become practical,^[9, 11] but today several library construction technologies have been demonstrated.^[12] A challenge in DECL synthesis is the compatibility of the chemical synthesis with the DNA itself and the biochemical steps (encoding and amplification). Here we push the limits of current chemical synthesis on DNA by performing a seven step synthesis of a DNA-encoded macrocycle library (DEML) that is inspired by polyketide and mixed peptide-polyketide natural products (see Fig. 1C & D).

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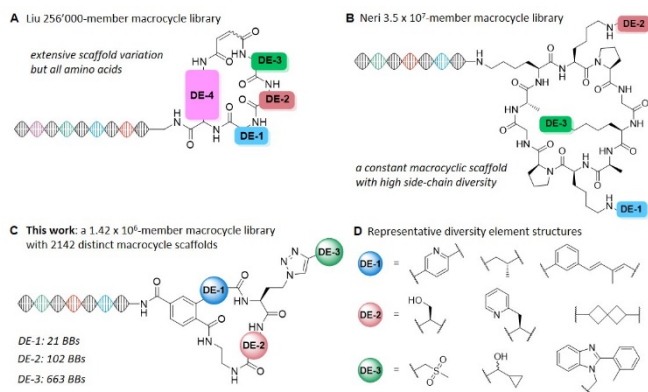


Figure 1. **A** The macrocycle library produced by the Liu lab is peptide derived, and rich in scaffold diversity.^[13] **B** The macrocycle library from the Neri lab looks at deep side-chain diversity derived from a single scaffold. **C** The library described here uses a DE-1 comprised exclusively of carbon or ether chains and a DE-2 that is a mix of carbon frameworks and amino acids. **D** Representative diversity element members.

The macrocycle synthesis on DNA. The building blocks of the two ring scaffold diversity elements (DE-1 & DE-2) were designed to cover a broad swath of ring structures with different sizes, shapes, strain and polarities (see the Electronic Supporting Information [ESI] for building blocks) Fig. 1C shows the general design of the DEML as well as a few representative examples of the building blocks (Fig. 1D). The synthetic starting point of the macrocycle ring was a readily available 2-iodoterephthalic acid derivative (**1** in Fig. 2A). In four chemical steps this precursor could be elaborated into the BBs for DE-1. Before embarking on the DEML synthesis we next carried out a full macrocycle synthesis of a single library member on a short single-stranded DNA so that we could optimize the chemistry at each step (Fig. 2B&C).

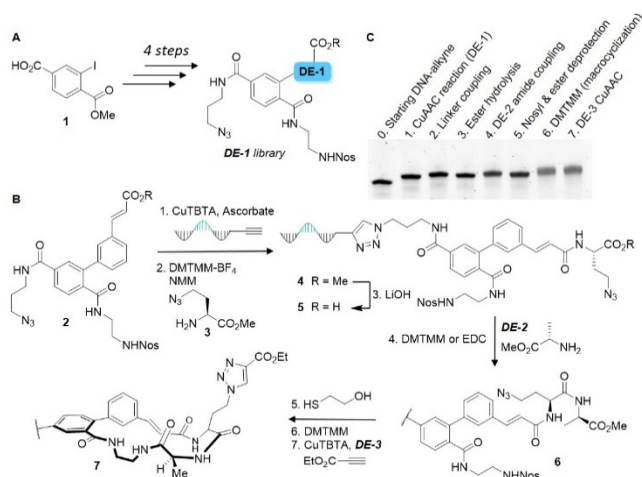


Figure 2. **A** The DE-1 library is created in four chemical steps from a readily available precursor. **B** A representative library member was synthesized completely on ssDNA to probe the compatibility with DNA. **C** The DNA remains pure throughout all seven steps of the macrocycle synthesis. Nos = 2-nitrophenylsulfonyl.

The synthesis began with a Cu-catalyzed azide-alkyne cycloaddition (CuAAC) to connect the DNA with the DE-1 library member **2** (step 1, Fig. 2B). The next step was to introduce the key trifunctional lynchpin **3** (step 2). This proved challenging because an α -azido- β -amino acid proved unstable, and azido-alanine led to elimination of the azide to create dehydroalanine under basic conditions. These two compact molecules were our preferred lynchpins because the aim was to keep the out-of-the-ring diversity element as close to the macrocyclic core as possible, since it would then have the greatest effect on the macrocycle's conformational space. In the end we had to settle for compound **3**, which contained an additional methylene spacer. Saponification of the methyl ester (step 3) then opened the necessary coupling site to install the DE-2 library. In the test synthesis we used alanine as a coupling partner and its coupling efficiency was highest with DMTMM (step 4). Given how critical this step was to the library we carried out a comprehensive screen across 126 amino acids and evaluated the reactions by LC-MS analysis. We scored the reactions by coupling efficiency and purity, applying thresholds of >80% conversion and \geq 50% product purity for inclusion in the DEML itself. Most amino acids exceeded these limitations, although we could identify trends in the poorly reacting members. In general, other than 1-aminocyclopropanecarboxylic acid and sarcosine, α,α -disubstituted and *N*-methylated amino acids were poorly coupled. Also problematic were homoprolines as well as compounds with leaving groups in the sidechain (chloroalanine, azidoalanine). The full list of the coupling results are shown in the ESI. The choice of the coupling reagent had a big impact on coupling efficiencies for the different building blocks. While EDC/HOAt/DIPEA is often the system of choice^[14] we found that coupling with DMTMM-BF₄/NMM was better in most cases, resulting in higher conversions and cleaner products. EDC/HOAt/DIPEA was only superior for strained cyclic secondary amino acids and tyrosine derivatives, a problem that likely stems from reaction of the tyrosine hydroxy group with DMTMM-BF₄/NMM. Informed by this reaction screening, in the library synthesis itself we coupled each DE-2 according to the optimal conditions identified in this screen. In total, after screening and applying the quality filters, a set of 102 different amino acids were selected for DE-2.

Prior to macrocyclization the ester and the 2-nitrophenylsulfonyl (Nos) group needed to be removed from **6** (step 5). This could be done in a one-pot operation using 2-mercaptoethanol (BME) in combination with DBU (step 5) to deliver the fully deprotected version of **6**. This seemingly simple step turned out to be tricky and dependent on the nature of the DE-2 moiety. In the ESI we describe a detailed optimization study across 20 representative macrocycle precursors with constant DE-1 and varying DE-2 (Figure Sxx). Most of the test compounds showed good deprotection efficiencies and low sideproduct formation. The best substrates were members with cyclic (proline derived) DE-2 elements. In contrast, amino acids with sterically hindered sidechains like adamantyl and dicyclohexyl were poorly deprotected. An unusual side-product was observed in certain cases, seemingly derived from cleavage of the trifunctional linker and the DE-2 moiety to leave the terminal amide of, for example, compound **2**. The azide is responsible for this side-reaction since if we synthesize a variant

of **4** with an alkyne instead of an azide, it does not occur. The Asn was also a poor performer in the Nos deprotection because the side-chain amide seemed to back-bite onto the backbone under the deprotection conditions. Nevertheless these undesired reactions affected only a small subset of the DE-2 members (particularly methylhistidine, serine) and hence we continued without further optimization. Macrocyclization (step 6) and the CuAAC (step 7) both occurred in high chemical yield and delivered pure product **7**, according to LC-MS analysis. As shown in the PAGE gel (Fig. 2C) the DNA shows no detectable degradation over all seven synthetic steps.

Split-and-pool synthesis of the library. With the synthetic route to the DEML established, the next task was to elaborate this into the full library. In DE-1 we included alkyl, aromatic, heteroaromatic and polyene moieties as well as combinations thereof. Since the DE-1 building blocks (BBs) needed to be synthesized individually we limited the number of members in this group to 21 (synthetic procedures and analytical data are found in the Supp. Info). As iterative cross-coupling strategies continue to evolve,^[15] we expect that DE-1 diversity in our scaffold could be expanded further. The DE-1 members were directly encoded by clicking each individual DE-1 with a unique 5'-hexynyl modified oligonucleotide (Figure Sxxx for complete library assembling procedure). The DE-1 library was pooled and split in 102 vessels and coupled with the DE-2s. The reactions were encoded by annealing with a partially complementary DNA strand, bearing the coding sequence for the introduced (second) diversity element in the 5'-overhang. After Klenow fill-in the vessels were pooled and purified to yield the DEML precursor library with 2142 encoded macrocycle precursors. These were deprotected with BME/DBU and macrocyclized with DMTMM at dilute concentration (10 μ M). Test macrocyclizations proposed at least 60% conversion and in most test cases we even found >80% conversion.

In preparation for introduction of the last diversity element the coding double-stranded DNA was restricted by BamHI, yielding a 5'-overhang of four bases on the non-macrocycle bearing strand. Despite extensive optimization, restriction yield could not be improved beyond 80% (see the ESI for further details). The restricted DEML was split into 663 vessels and coupled with an alkyne library. The CuAAC was performed at 2.5 μ M concentration and so we used large excesses of alkyne (100 eq.), copper catalyst (200 eq.) and ascorbate (200 eq.) to obtain full conversion. The individual reactions were subsequently encoded by T4 ligation and Klenow fill-in with a pre-annealed partially double-stranded DNA strand, showing a 5'-phosphorylated GATC overhang and the six-bases codon for the DE-3 (see SupplInfo for detailed coding strategy). Purification of the DEML was performed by reversed phase column chromatography.

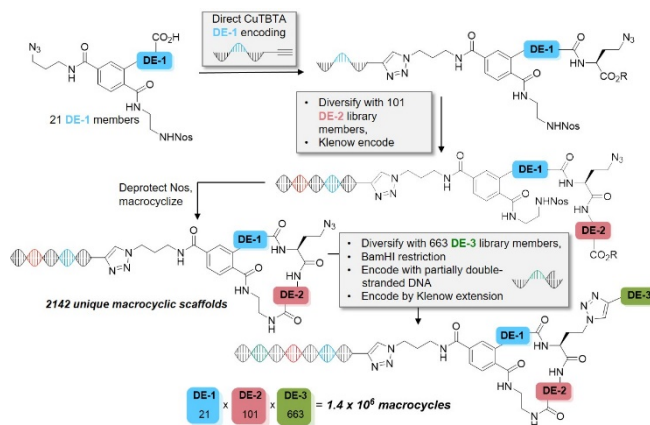


Figure 3. Complete library synthesis and encoding strategy. The detailed chemical steps are outlined in Figure 2.

Prior to protein binding assays all theoretical members of our library were analyzed for their physicochemical properties with the RDKit (www.rdkit.org) package, which included a custom script to interface with our library tables (described in the ESI). We compared the results with the published guidelines for oral macrocyclic drugs recently defined by the Whitty^[32] and Kihlberg^[6, 16] groups (**Figure 4**). Our library falls largely within the reported guidelines since the majority of the members showed a molecular weight below 1000 Da, a total polar surface area (TPSA) between 150 and 250 \AA^2 and an estimated partition coefficient (AlogP) between 0 and 6. Furthermore the diversity of our macrocycle scaffolds can be seen by the ring-size distribution. While the majority of the members consists of 17 - 23 ring atoms, the range is from 16 up to 33 ring atoms. Further property analyses and comparison to the published guidelines are shown in the ESI.

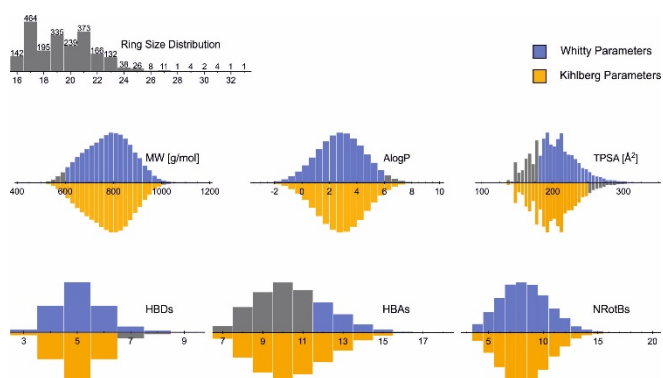


Figure 4. Molecular property distribution across the entire 1.4×10^6 -membered DEML library. Blue/orange indicate the region found favorable for oral bioavailability of known macrocyclic drugs according to published guidelines.

After these evaluations of the DEML we used it for selections against two human blood serum proteins, human serum albumin (HSA) and α -1-acid glycoprotein (AGP). HSA is the most abundant protein in human blood serum and carries mostly acidic ligands.^[17] AGP is a second, less abundant transport protein in human blood serum, and it carries mainly neutral and

basic molecules such as the macrocyclic polymyxins (e. g. Colistin).^[18] Protein binding assays were performed on hydrophilic streptavidin-coated magnetic beads, which were pretreated with biotinylated HSA and AGP respectively.^[19] The DNA of the eluted binders from the treatments was amplified by PCR and analyzed by next generation high throughput sequencing in comparison with library fingerprint and bead only (dummy) selections. The resynthesized hits from HSA selections delivered mid-high micromolar binding and were not pursued further (full discussion in the ESI). In the case of AGP binding several hits were selected for resynthesis (see ESI for detailed synthetic procedures). The top two hits, which appeared as singleton hits, gave low or poor binding in differential scanning fluorimetry (see the ESI) assays. The fourth highest hit contained the most highly represented DE-2 in the top 100 hits, and indeed showed a strong thermal denaturation shift in a differential scanning fluorimetry assay. Detailed characterization by isothermal titration calorimetry (ITC) revealed a low micromolar dissociation constant ($K_D = 7 \mu\text{M}$, Figure 6c). Intriguingly, a similar binding constant ($K_D = 4 \mu\text{M}$) was determined when we tested the azide precursor (Figure 6d), suggesting that the macrocyclic scaffold, and not the DE-3 side-chain, was the key for good binding. This result reveals that the azide could be a starting point for further improvement of the binder. For example, starting from the macrocycle with only DE-1 and DE-2 installed (see X in Fig. 3), a Staudinger reduction followed by reductive amination with aldehydes would provide an amine sub-library pre-enriched in strong binders. A great advantage of the modular synthetic design means such a strategy could be done with library precursors that were already prepared.

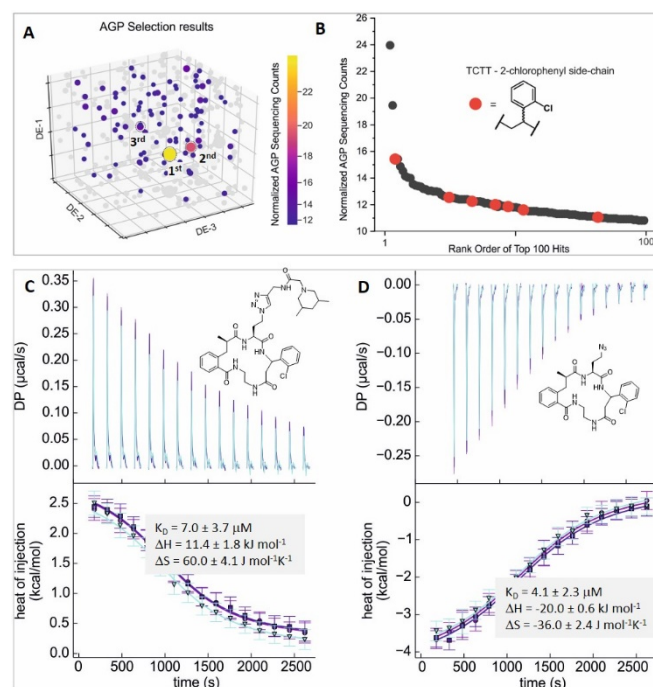


Figure 5. **A** The three top hits from AGP selection results were resynthesized. **B** The TCTT codon, corresponding to the 2-chlorophenyl side-chain, was the most strongly selected codon in the top 100 hits. **C** Isothermal titration calorimetry reports a $K_D = 7 \mu\text{M}$. **D** The azide precursor to the selected

macrocycle shows a similarly potent binding constant ($K_D = 4 \mu\text{M}$), indicating that the macrocycle scaffold is the key binding feature.

We have synthesized and characterized a macrocycle library bearing hydrophobic backbone elements reminiscent of macrocyclic polyketide natural products. The greatest challenge was the synthesis of the largely hydrocarbon DE-1 diversity elements. Although only 21 members, this sub-library preparation was the most time-consuming part of the synthesis, highlighting the need for continued development of carbon-carbon bond-forming reactions compatible with DNA encoded library construction. Although over a million members, a better way to conceptualize this library is that it contains 2142 macrocyclic scaffolds each with 663 side-chain variations. It is therefore encouraging that even with such a relatively small number of scaffolds, a low micromolar hit could be identified in a protein selection. Scaffold diversification is a great challenge in all types of library preparation and our results reveal this is no different for macrocycles. While the current state of synthesis will dictate that encoded libraries are dominated by a single core structure with diverse side-chains, iterative synthetic strategies^[15, 20] compatible with DNA would open new areas of chemical space to DECL. Our results with the DEML reported here suggest this to be a compelling pursuit for organic synthesis.

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Keywords: macrocycles • DNA encoded libraries • Lipinski rules • DNA chemistry • chemical library

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Supporting Information

A DNA-encoded macrocycle library that resembles natural macrocycles

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Table of Contents

1. General Information

1.1 General Experimental Information

Reagents, Solvents, Oligonucleotides

Reagents and solvents used were purchased from Sigma-Aldrich, Alfa Aesar, Apollo Scientific Ltd., Fluka, Fluorochem, Enamine, TCI, Bachem, Biosolve or Acros and used as received. Enzymes and their buffers were purchased from New England Biolabs. Oligonucleotides were purchased from Microsynth in desalted or HPLC purified form as lyophilized material or dissolved in H₂O as 100 μ M stock solutions. MOPS buffer refers to a solution of 50 mM MOPS 500 mM NaCl in H₂O, pH 8.2.

Chromatographic Purification and Isolation

Flash chromatography was performed on SilicaFlash® gel P60 40-63 μ m (230-400 mesh) (SiliCycle, Quebec) according to Still^[1] or on a Biotage Isolera four using SilicaFlash® gel packed cartridges. Reversed phase chromatographic separations were conducted on a Biotage Isolera four, using LiChroprep RP-18 (40-63 μ m) silica from Merck. Preparative RP-HPLC was carried out on a Shimadzu Prominence UFLC Preparative Liquid Chromatograph.

Method A: Gemini NX-C₁₈, 5 μ m, 110 Å, 21.2 x 250 mm from Phenomenex with a flow rate of 20 mL/min, gradient: 1% (3 min)-99% (25 min)-99% (3 min) (B), monitoring and collecting the products at 254 nm. Buffer (A): 0.1% TFA (v/v) in H₂O, Buffer (B): 0.1% TFA (v/v) in MeCN.

Method B: Gemini NX-C₁₈, 5 μ m, 110 Å, 21.2 x 250 mm from Phenomenex with a flow rate of 20 mL/min, gradient: 1% (3 min)-80% (25 min)-99% (0.1 min)-99% (3 min) (B), monitoring and collecting the products at 254 nm. Buffer (A): 50 mM TEAA in H₂O, pH 7.2, Buffer (B): MeCN.

Method C: Jupiter C₄, 5 μ m, 300 Å, 10 x 250 mm from Phenomenex with a flow rate of 10 mL/min, gradient: 0% (3 min)-30% (17 min)-30% (2 min) (B), monitoring and collecting the products at 254 nm. Buffer (A): 50 mM TEAA in H₂O, pH 7.2, Buffer (B): MeCN.

The crude compound mixtures were injected as H₂O, MeCN, MeOH or DMSO solutions. Buffers and HPLC eluents were prepared with nanopure water (resistivity 18.2 M Ω). Concentration under reduced pressure was performed by rotatory evaporation at 40°C. Aqueous product fractions were frozen in liquid N₂ and lyophilized on a Christ Alpha 2-4 LDplus flask lyophilizer at 0.3 mbar or below.

Chromatographic Analysis

Analytical TLC was performed on Silica gel 60 F254, 0.25 mm pre-coated glass plates (Merck) and visualized by fluorescence quenching under UV light at 254 nm and subsequent KMnO₄ or ninhydrin staining.

HPLC analysis was performed on an Agilent 1100 system equipped with Jupiter C₄, 5 μ m, 300 Å, 2 x 50 mm or 2 x 150 mm columns with a flow rate of 0.6 mL/min. Gradients: 0% (1.8 min)-30% (3.2 min)-90% (2.2 min)-90% (1.8 min) (B) or 0% (1.8 min)-30% (12.2 min)-90% (4 min)-90% (2 min) (B), Buffer (A): 50 mM NH₄OAc in H₂O, pH 7.2, Buffer (B): MeCN.

ESI-MS and LC-MS

ESI-MS spectra were recorded on a Bruker Esquire3000 spectrometer by direct injection in positive or negative polarity of the ion trap detector. Compounds were injected as MeOH, MeCN or H₂O solutions. High-resolution mass spectra (HRMS) were recorded by the mass spectrometric service of the University of Basel on a Bruker maXis 4G QTOF ESI mass spectrometer.

LC-MS spectra were recorded on a hyphenated system, consisting of the previously described Agilent 1100 HPLC and the Bruker Esquire3000 ESI-MS. Hyphenation software: HyStar 3.1.

UPLC-MS was carried out on an Agilent 1290 Infinity system equipped with an Agilent 6130 Quadrupole ESI-MS using a C₁₈ column (ZORBAX Eclipse Plus RRHD, 1.8 μ m, 2.1 x 50 mm) from Agilent with a flow rate of 0.45 mL/min at 40°C. Buffer (A): 0.1% (v/v) formic acid in H₂O/1% MeCN (v/v), Buffer (B): 0.1% (v/v) formic acid in MeCN/1% H₂O (v/v) using the following gradient: 5-90% (3.5 min)-90% (1 min) (B), ESI-MS in positive ion mode of the ion trap.

Nuclear Magnetic Resonance (NMR)

¹H, ¹³C and 2D-NMR spectra were acquired on a BrukerAvance (400, 500 or 600 MHz proton frequency) spectrometer at 298.15 K. Chemical shifts (δ values) are referenced to the solvent's residual peak and reported in ppm. Multiplicities are reported as follows: s = singlet, sbr = broad singlet, d = doublet, t = triplet, dd = doublet of doublets, td = triplet of doublets, m = multiplet or unresolved and coupling constant J in Hz.

DNA Purification/Handling

0.2, 0.5, 1.5 and 2.0 ml tubes were centrifuged in an Eppendorf Centrifuge 5418R. 5, 14 and 45 ml tubes as well as 96-well plates were centrifuged in an Eppendorf Centrifuge 5804R. DNA and protein sample heating or cooling was performed with a BIOER Mixing Block MB-102. Vacuum centrifugation was performed with an Eppendorf Concentrator 5301. DNA and protein concentration measurements were performed on a Nanodrop 2000 from Thermo Scientific.

Gel Electrophoresis

Gel electrophoresis was performed with a Bio-Rad PowerPac HV high-voltage power supply. Gels were prepared in 83 x 83 x 1.0 or 1.5 mm format with 10 or 15 wells.

Native DNA polyacrylamide gels were prepared from 40% acrylamide/bis-acrylamide 19:1 (Fisher Scientific) with TBE (TRIS-Borate-EDTA) buffer with 0.1% (v/v) TEMED (*N,N,N',N'*-Tetramethylethylenediamine-1,2-diamine) and 0.1% (v/v) APS (25% ammonium persulfate in H₂O solution). Loading dye: Gel loading dye purple (6X), no SDS from NEB. To improve loading, the dye was used as 3X.

For denaturing DNA polyacrylamide gels the same recipe was used with the addition of urea (final concentration: 7M). The DNA sample was treated with 2X formamide loading dye (95% formamide, 5 mM EDTA, pH 8, 0.025% (m/v) bromophenol blue) and denatured at 95°C for 2 min prior to loading onto the gel.

DNA agarose gels were prepared by heat dissolving agarose (Fisher Scientific) in 1X TBE (50 ml per gel) and cooling to room temperature. Loading dye: 0.025% (m/v) bromophenol blue in 30% (v/v) glycerol in TE buffer, pH 8.

Denaturing protein gels were prepared with a 5% stacking gel (approx. 2 cm high) and x% resolving gel. The stacking gel was prepared from 40% acrylamide/bis-acrylamide 37.5:1 (Fisher Scientific) with 0.1% (m/v) SDS, 0.2% (v/v) TEMED and 0.4% (v/v) APS (10% APS solution in H₂O) in 125 mM TRIS, pH 6.8. The resolving gel was prepared from 40% acrylamide/bis-acrylamide 37.5:1 (Fisher Scientific) with 0.1% (m/v) SDS, 0.1% (v/v) TEMED and 0.3% (v/v) APS (10% APS solution in H₂O) in 375 mM TRIS, pH 8.8. Protein samples were treated with 2X loading dye (66 mM TRIS pH 6.8, 2% (m/v) SDS, 0.01% (m/v) bromophenol blue, 30% (v/v) glycerol) and denatured at 95°C for 5 min prior to gel loading. Running buffer for DNA gels: 1X TBE. Running buffer for protein gels: 193 mM glycine, 25 mM TRIS, 0.1% (m/v) SDS.

PCR and qPCR

PCR was performed in 0.2 ml PCR tubes in a Bio-Rad T100 Thermal cycler. qPCR was performed in a StepOnePlus real-time PCR system from Applied Biosystems using StepOne v2.3 software. qPCR samples were set up in 96-well plates.

Differential Scanning Fluorimetry (DSF) and Isothermal Calorimetry (ITC)

Thermal denaturation experiments were performed with a Nanotemper Prometheus NT.48 instrument using standing capillaries. All samples were subjected to continuous ramping from 20-95°C at a rate of 1.5°C/min. The thermal denaturation was monitored by the intrinsic fluorescence emission at 330/350 nm after excitation at 285 nm. The data was processed using a beta-version of the Nanotemper PR.Analysis software and Graphpad Prism v7.

ITC experiments were performed on an ITC200 instrument from Malvern Panalytic. Protein samples were degassed prior to ligand binding assays. The ligand solution in the syringe was added stepwise to the protein solution in the sample cell at 10 or 25°C. 300 s initial delay with stirring was followed by 17 syringe injections (1 x 0.5 µL, 16 x 2.3 µL). The baseline subtraction and integration of the differential power vs. time was performed using NITPIC^[2] and data fitting was performed using Sedphat.^[3]

1.2 LC-MS Analysis of DNA-tagged Small Molecules

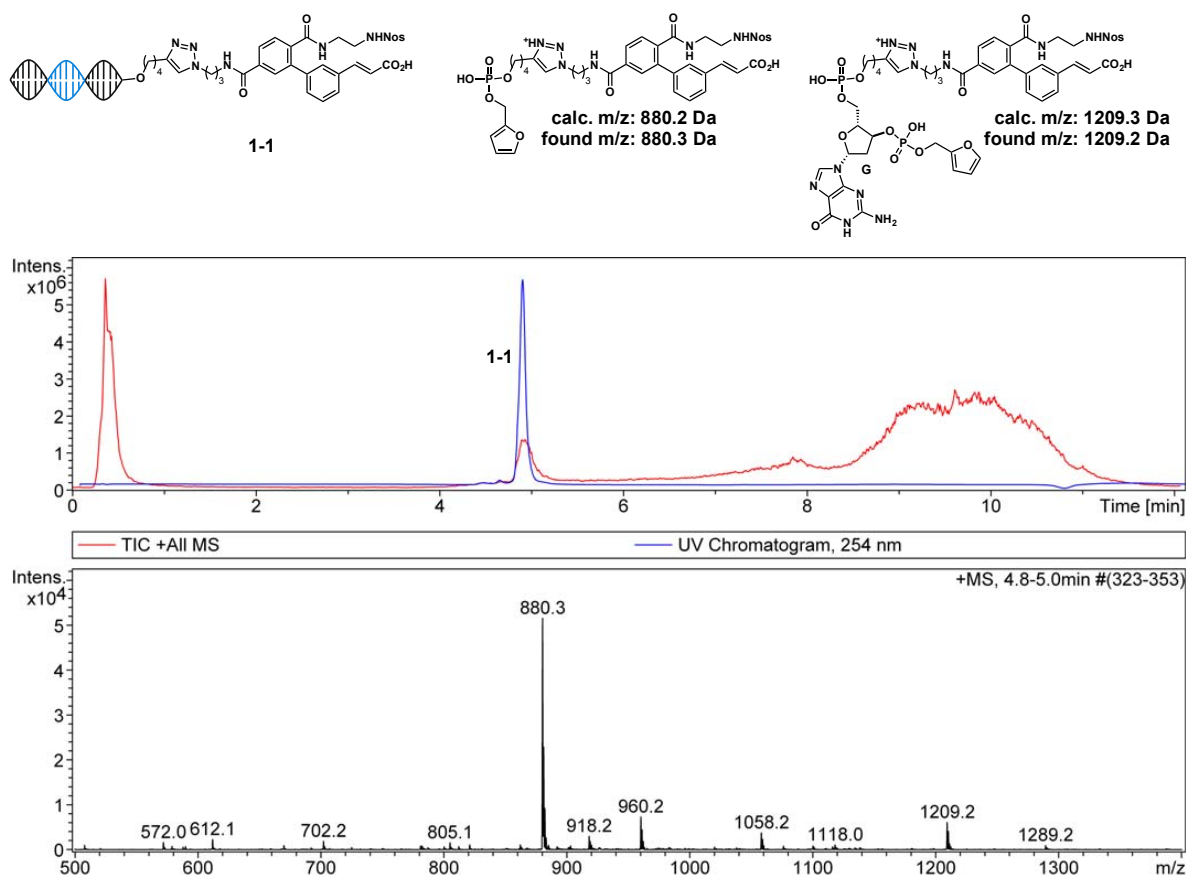


Figure 1. Example of an encoded macrocycle precursor LC-MS analysis with the found ion fragments

Chemical modifications of DNA-tagged small molecules were analyzed with a hyphenated system consisting of an Agilent 1100 HPLC and a Bruker Esquire3000 ESI-MS with a direct connection tube between the devices (no flow splitter). The ESI-MS was run at 350°C with a N₂ flow of 10.5 L/min and 35 psi pressure in positive ionization mode. Tuning ranges used were 500 - 1400 or 1000 - 1800 Da. Control software for the ESI-MS was Esquire Control and for the hyphenated system HyStar 3.1 was used. With this setup DNA-encoded compounds could be analyzed due to the specific fragmentation of the molecule. Therefore, no multiply charged species needed to be deconvoluted, which made it possible to directly find 1 Da modifications of the attached small molecule. The accuracy of this method was determined to be ≤ 0.3 Da. In **Figure 1** an example of an encoded macrocycle precursor is shown. The MS trace analysis clearly shows the proposed fragments. The major fragment consists of the small molecule with the phosphate and the ribose (now as furyl group) of the first nucleotide. The rest of the DNA strand was eliminated. A second less abundant fragment has the same structure but the elimination occurred at the second nucleotide whereby the first nucleotide (in our case G) stayed intact. Sodium ($M + Na^+$) and potassium ($M + K^+$) adducts were often found along with the protonated species.

1.3 DNA Ethanol Precipitation

DNA samples were treated with 3M NaOAc pH 5.2 buffer (10% of DNA sample volume) and mixed with 3-4 volumes EtOH. The mixture was kept on ice for 2 h (unless otherwise stated). The DNA suspension was centrifuged (4°C, 16900 g, 30 min) and the supernatant was discarded. The obtained pellet was washed twice with cold EtOH and centrifuged again (4°C, 16900 g, 2 x 15 min). The supernatants were discarded and the washed pellet was dried in the air for 30 min. Clean pellets were dissolved in H₂O or buffer.

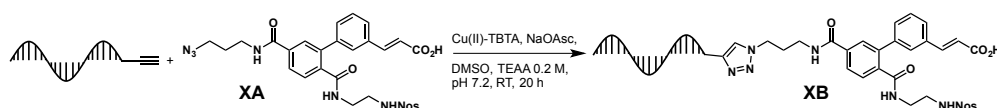
1.4 10 mM Cu(II)-TBTA Stock Solution in 55% DMSO

The Cu(II)-TBTA stock solution was prepared according to a published procedure for click modifications of oligonucleotides found on the Lumiprobe website (<https://www.lumiprobe.com/protocols/click-chemistry-dna-labeling>).

A solution of copper(II) sulfate pentahydrate (50 mg, 200 μ mol 1.0 eq.) in distilled H₂O (10 mL) was mixed with a solution of TBTA (116 mg, 219 μ mol, 1.1 eq.) in DMSO (11 mL). The dark blue solution was stored at room temperature for months without any observed loss of catalytic activity in copper-catalyzed click reactions.

2. Test Synthesis of a DNA-Encoded Macrocycle

2.1 Synthesis of the Encoded Macrocycle Precursor **XB** by Click Reaction with Alkyne-Modified DNA



In a 1.5 ml Eppendorf tube a 5'-alkyne-DNA strand (1 mM in H₂O, 44.0 μ L, 1.0 eq.) of the sequence 5'-hexyne-GGA GCT TGT GAA TTC TGG ATG GGA CGT GTG TGA ATT GTC-3', DE-1 azide **XA** (10 mM in DMSO, 8.8 μ L, 2.0 eq.), sodium ascorbate (5 mM in H₂O, 44.0 μ L, 5.0 eq.), TEAA buffer (1M, pH 7.2, 88 μ L), DMSO (198 μ L) and H₂O (35 μ L) were mixed. N₂ was bubbled through the solution for 30 s. The Cu(II)-TBTA solution (10 mM in 55% DMSO, 22.0 μ L, 5.0 eq.) was added and the solution was degassed again for 30 s. The mixture was agitated at RT for 20 h after which HPLC showed 88% conversion to the desired product. The mixture was purified by preparative HPLC (Method B). The product containing fractions were combined and lyophilized to yield the desired product **XB** as a white solid. The DNA was dissolved in MOPS buffer (161 μ L) to yield a clear solution (140 μ M, **51%**).

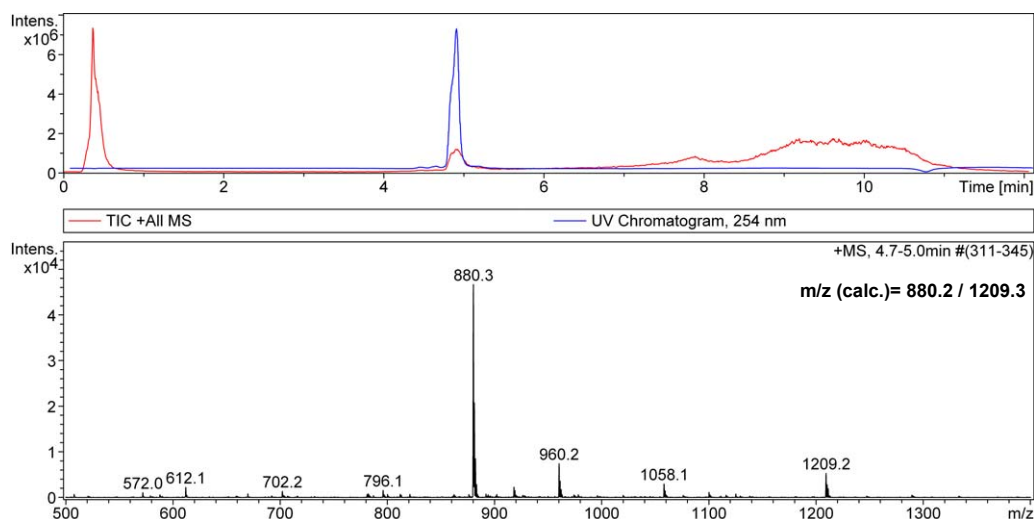
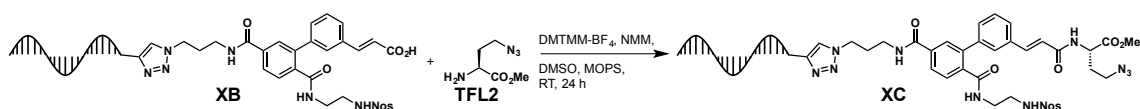


Figure 2. LC-MS chromatogram of the purified encoded compound **XB**.

2.2 TFL2 Amide Coupling with **XB**



In a 1.5 ml Eppendorf tube **XB** (140 μ M in MOPS buffer, 21.4 μ L, 1.0 eq.) was mixed with **TFL2** (0.5 M in DMSO, 6.0 μ L, 1000 eq.), DMTMM-BF₄ (400 mM in DMSO, 7.5 μ L, 1000 eq.) and NMM (400 mM in DMSO, 15.7 μ L, 2100 eq.). The mixture was agitated at RT for 24 h. The reaction was purified by EtOH precipitation and the clean DNA pellet was dissolved in H₂O (25 μ L) to yield the desired product **XC** as a clear solution (130 μ M, **quant.**).

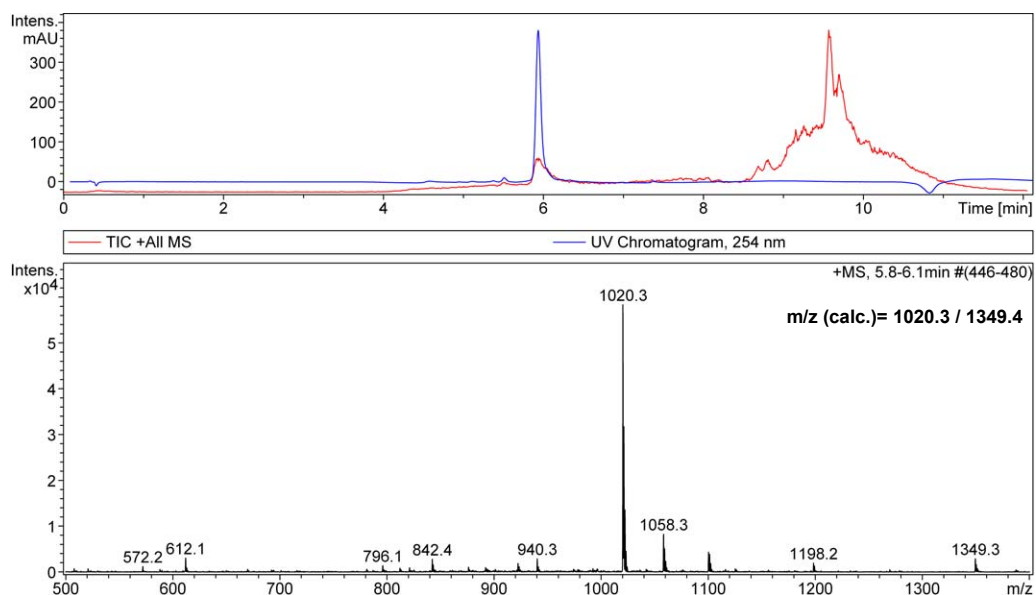
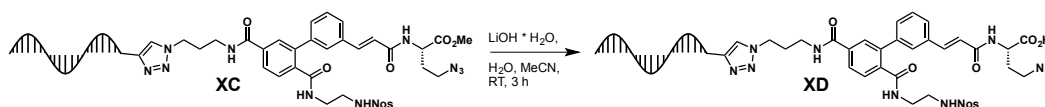


Figure 3. LC-MS chromatogram of the purified encoded compound **XC**.

2.3 Ester Hydrolysis of **XC**



In a 1.5 ml Eppendorf tube **XC** (130 μM in H_2O , 25.0 μL , 1.0 eq.), $\text{LiOH} \cdot \text{H}_2\text{O}$ (100 mM in H_2O , 4.9 μL , 150 eq.) and MeCN (15 μL) were mixed and agitated at RT for 3 h. The reaction was purified by EtOH precipitation and the clean DNA pellet was dissolved in MOPS buffer (28 μL) to yield the desired product **XD** as a clear solution (100 μM , **86%**).

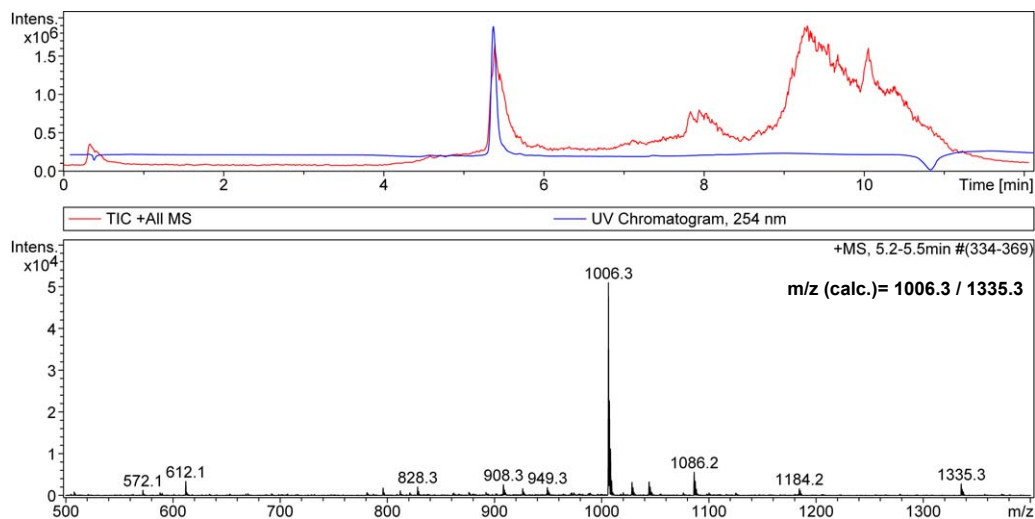
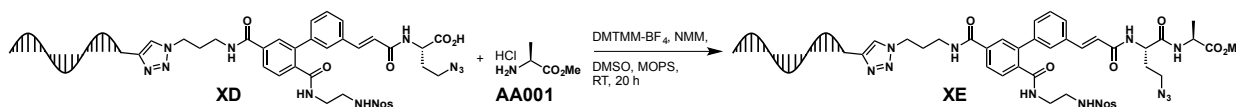


Figure 4. LC-MS chromatogram of the purified encoded compound **XD**.

2.4 Amide Coupling with **AA001**, Introduction of the 2nd Diversity Element



In a 1.5 ml Eppendorf tube **XD** (90 μM in MOPS buffer, 29.0 μL , 1.0 eq.) was mixed with *L*-alanine methyl ester hydrochloride **AA001** (500 mM in DMSO, 5.2 μL , 1000 eq.), DMTMM- BF_4 (150 mM in DMSO, 17.4 μL , 1000 eq.) and NMM (250 mM in DMSO, 36.5 μL , 3500 eq.) and the solution was agitated at RT for 20 h. The mixture was purified by EtOH precipitation and the clean DNA-pellet was dissolved in H_2O (135 μL) to yield the desired product **XE** as a clear solution (20 μM , **quant.**).

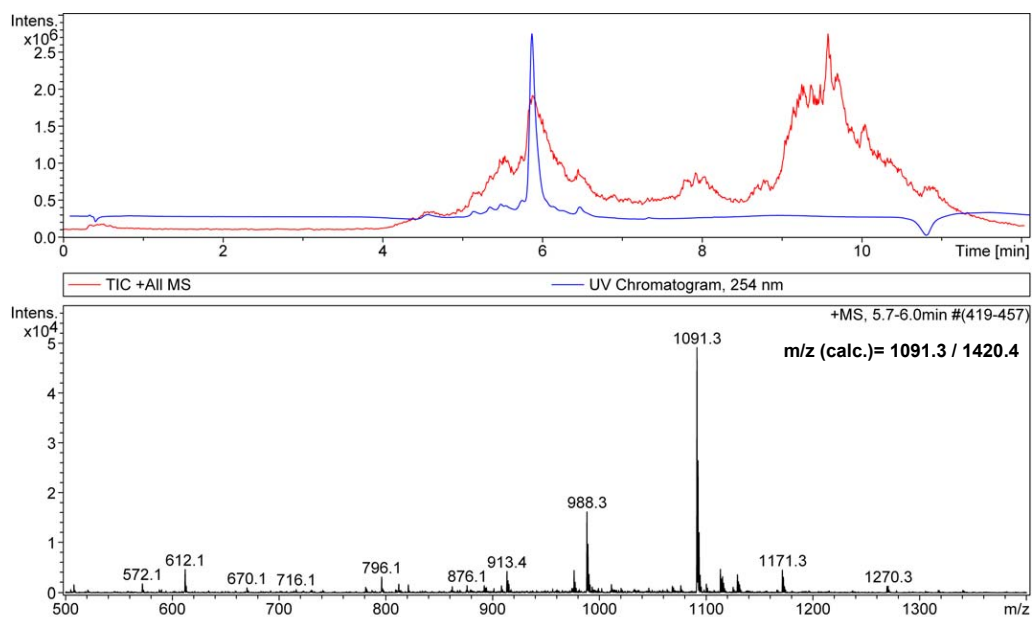


Figure 5. LC-MS chromatogram of the purified encoded compound **XE**.

2.5 Ester Hydrolysis and NOSYL Deprotection of **XE**



In a 0.5 ml Eppendorf tube **XE** (50 μ M in 300 mM MOPS/500 mM NaCl pH 8.2 buffer, 15.0 μ L, 1.0 eq.) was mixed with DBU (300 mM in DMSO, 15.0 μ L, 6000 eq.) and BME (300 mM in DMSO, 15.0 μ L, 6000 eq.). The mixture was degassed with N_2 for 30 s and agitated at RT for 6 h. The reaction was purified by EtOH precipitation and the clean DNA pellet was dissolved in MOPS buffer (20 μ L) to yield the desired product **XF** as a clear solution (30 μ M, **80%**).

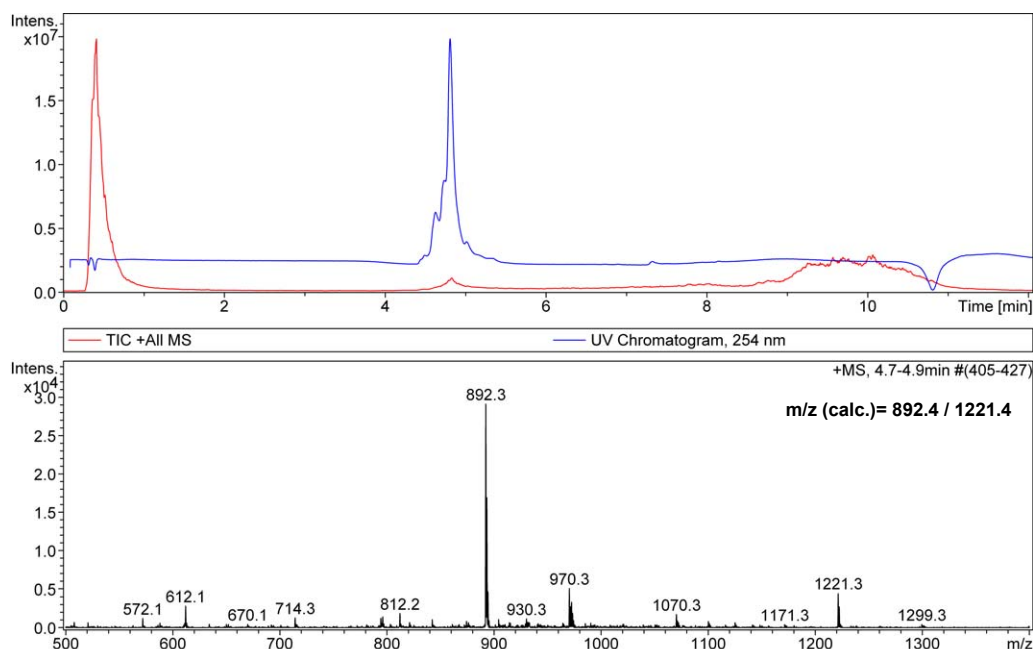
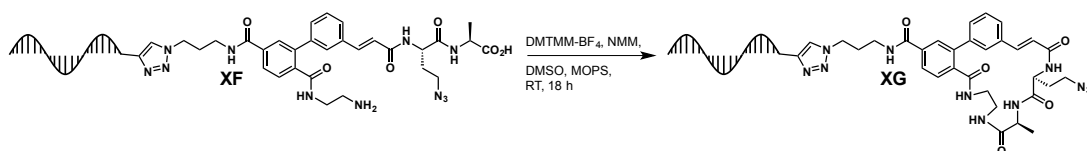


Figure 6. LC-MS chromatogram of the purified encoded compound **XF**.

2.6 Macrocyclization of **XF**



In a 0.5 ml Eppendorf tube **XF** (25 μ M in MOPS buffer, 13.0 μ L, 1.0 eq.) was mixed with MOPS buffer (2.0 μ L), DMSO (4.6 μ L), DMTMM-BF₄ (25 mM in DMSO, 3.9 μ L, 300 eq.) and NMM (50 mM in DMSO, 6.5 μ L, 1000 eq.). The mixture was agitated at RT for 18 h. The reaction was purified by EtOH precipitation. The reaction was set up again with above stated reagent amounts. After EtOH precipitation the clean DNA pellet was dissolved in H₂O (15 μ L) to yield the desired product **XG** as a clear solution (24 μ M, **quant.**)

Note: The macrocyclization was repeated once because LC-MS showed incomplete conversion. Increase of the coupling reagent amount (up to 500 eq.) and DMSO amount (>55%) should yield full macrocyclization in one step.

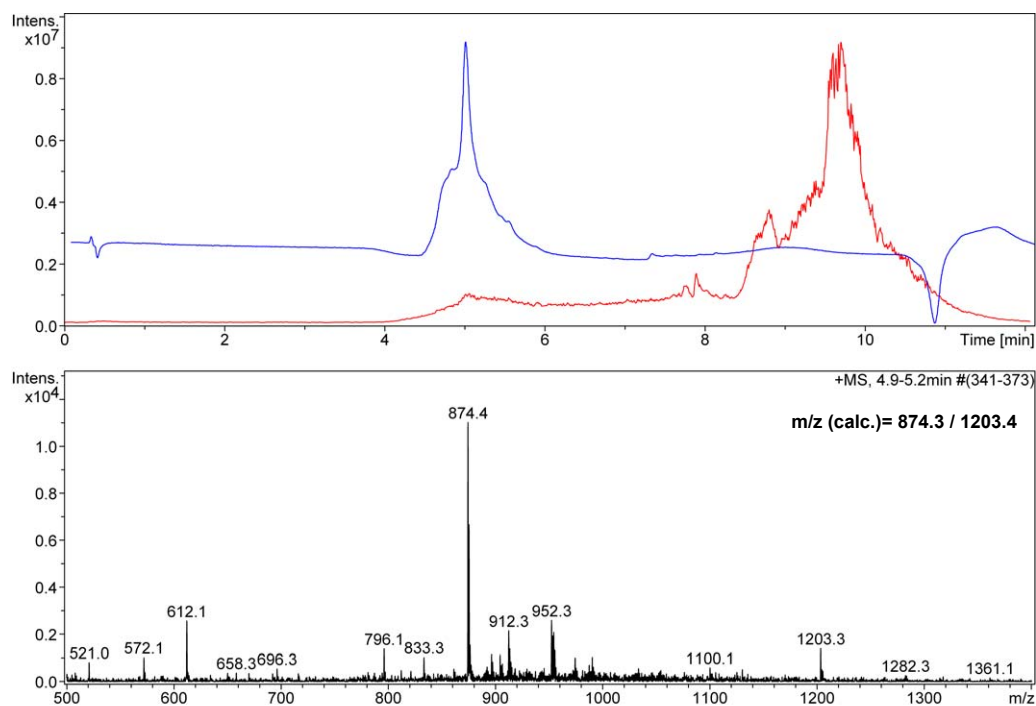
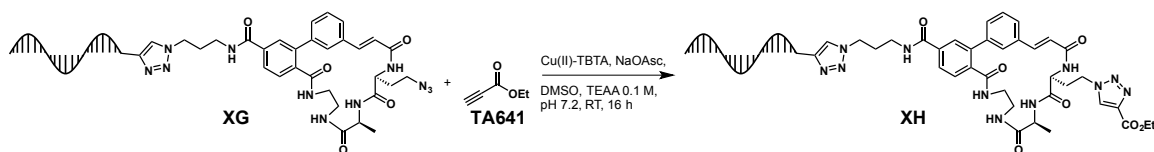


Figure 7. LC-MS chromatogram of the purified encoded compound **XG**.

2.7 Diversity Element 3 Introduction by Click Reaction



In a 0.5 ml Eppendorf tube, **XG** (10 μ M in 500 mM TEAA pH 7.2, 26.4 μ L, 1.0 eq.) was mixed with ethyl propiolate **TA641** (1 mM in DMSO, 26.4 μ L, 100 eq.), sodium ascorbate (3 mM in H₂O, 17.6 μ L, 200 eq.) and DMSO (17.5 μ L) and degassed with N₂ for 30 s. Copper(II)-TBTA complex (3 mM in 55% DMSO, 17.6 μ L, 200 eq.) was added and the solution was degassed with N₂ again. The solution was agitated at RT for 16 h. The reaction was purified by EtOH precipitation and the clean DNA pellet was dissolved in H₂O (15 μ L) to yield the desired product **XH** as a clear solution (14 μ M, **80%**).

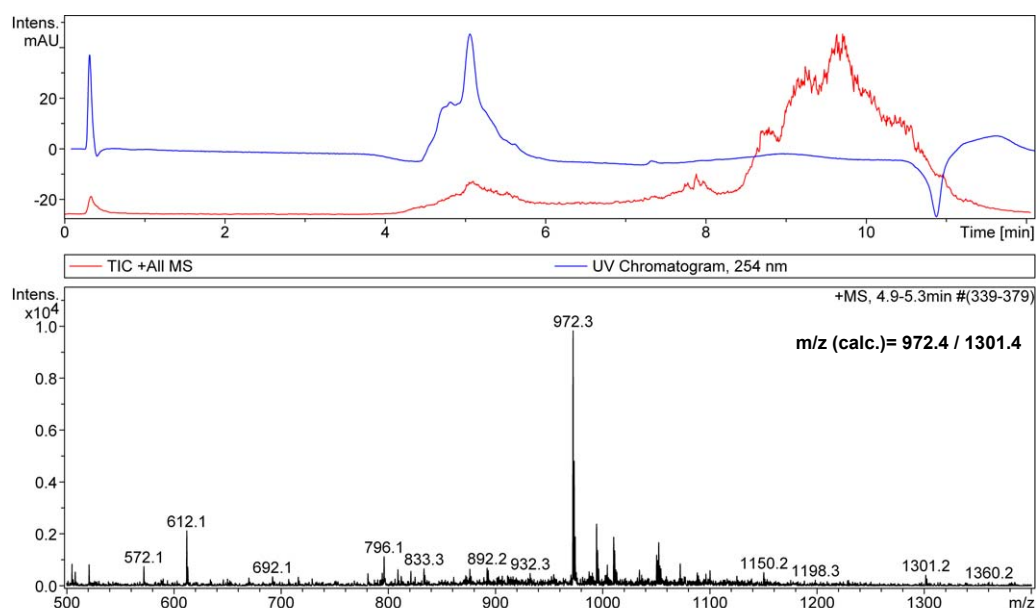
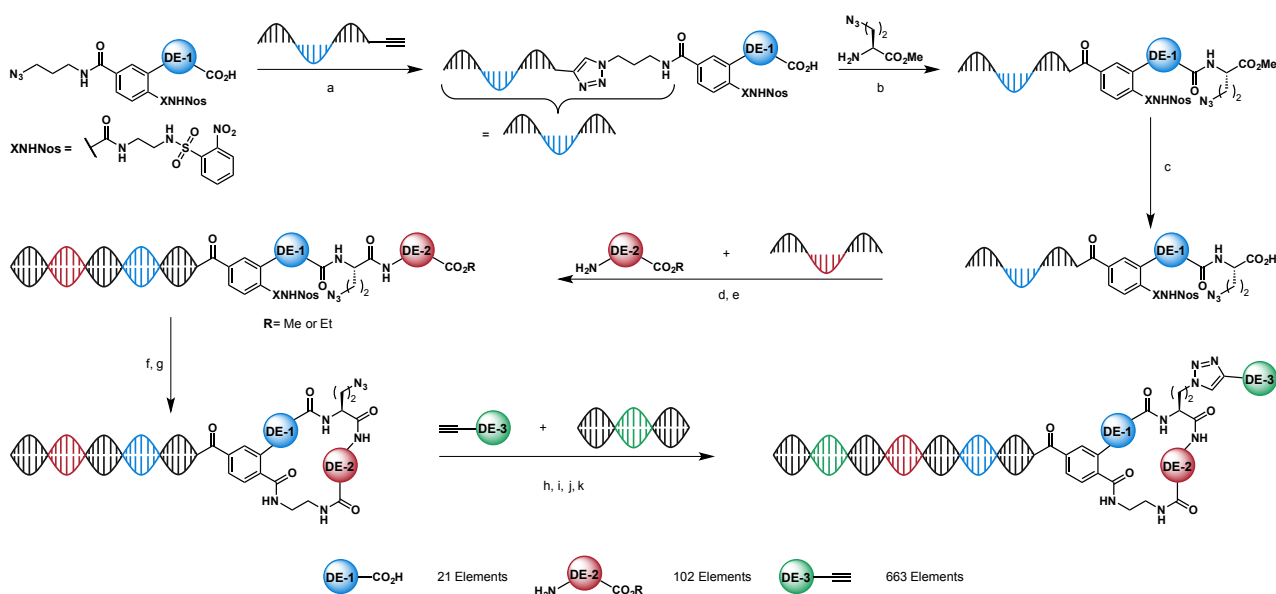


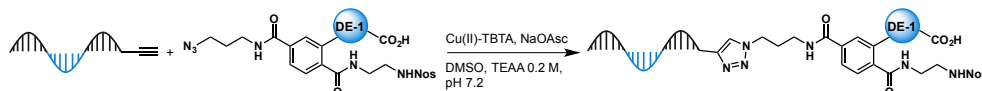
Figure 8. LC-MS chromatogram of the purified encoded compound XH.

3. DNA-Encoded Macrocycle Library Synthesis



Scheme 1. DEML synthesis overview. **a)** Cu-TBTA, NaOAsc, TEAA buffer pH 7.2, DMSO, RT, 20 h. **b)** DMTMM-BF₄, NMM, MOPS buffer pH 8.2, DMSO, RT, 24 h. **c)** LiOH, H₂O/MeCN 3:1, RT, 2.5 h. **d)** DMTMM-BF₄, NMM, MOPS buffer pH 8.2, DMSO, RT, 20 h or EDC·HCl, HOAt, DIPEA, MOPS buffer pH 8.2, DMSO, RT, 20 h. **e)** Klenow Polymerase, NEBuffer 2, dNTPs, 25°C, 30 min. **f)** 2-Mercaptoethanol, DBU, MOPS buffer pH 8.2, DMSO, RT, 6 h. **g)** DMTMM-BF₄, NMM, MOPS buffer pH 8.2, DMSO, RT, 18 h. **h)** BamHI-HF, CutSmart buffer, 37°C, 30 min. **i)** Cu-TBTA, NaOAsc, TEAA buffer pH 7.2, DMSO, RT, 16 h. **j)** T4 DNA ligase, NEB ligase buffer, ATP, 16°C, 16 h. **k)** Klenow Polymerase, NEB ligase Buffer, dNTPs, 25°C, 30 min.

3.1 Encoding of the Diversity Elements 1 (DE-1) with Alkyne-Modified DNA (21 elements)



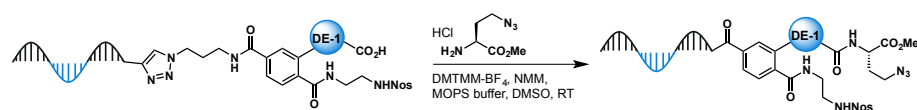
In a 1.5 ml Eppendorf tube an alkyne-DNA strand (1 mM in H₂O, 1.0 eq.) of the sequence 5'-hexyne-GGA GCT TGT GAA TTC TGG XXX GGA CGT GTG TGA ATT GTC-3', DE-1 azide (10 mM in DMSO, 2.0 eq.), sodium ascorbate (5 mM in H₂O, 5.0 eq.), TEAA buffer (1M, pH 7.2), DMSO and H₂O were mixed. N₂ was bubbled through the solution for 30 s. Cu(II)-TBTA complex (10 mM in 55% DMSO, 5.0 eq.) was added, N₂ was bubbled through the solution again and the mixture was shaken at RT overnight (20 h). The final concentrations of the reagents were as follows: 100 μM DNA, 200 μM DE-1 azide, 500 μM Cu(II)-TBTA complex, 500 μM sodium

ascorbate, 0.2 M TEAA, 50% DMSO. Typical DNA amounts: 30 nmol. Conversion and purity of the product was measured by HPLC. The mixture was directly purified by prep. HPLC Method B. Product-containing fractions were combined and lyophilized. The isolated product was dissolved in MOPS buffer to yield a 140 μ M solution. Typical conversions: 80-90%. XXX represents the three-base codon for the diversity element 1. This procedure was repeated for all DE-1 (Table 1).

Table 1. All 21 diversity element 1 building blocks. R indicates the constant scaffold.

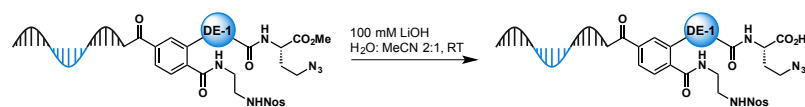
No.	Structure	No.	Structure	No.	Structure
NP01		NP08		NP15	
NP02		NP09		NP16	
NP03		NP10		NP17	
NP04		NP11		NP18	
NP05		NP12		NP19	
NP06		NP13		NP20	
NP07		NP14		NP21	

3.2 Trifunctional Linker Coupling with DNA-DE-1 Pool



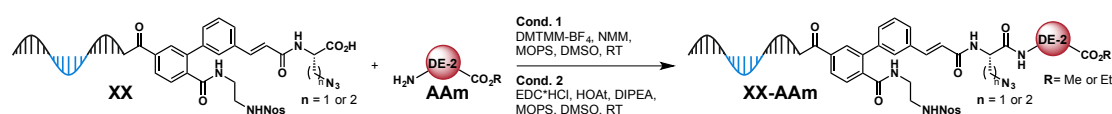
20 encoded DE-1 DNA strands (NP1-6 and NP8-21; 21.5 μ L, 3 nmol each, 50 mM MOPS buffer) were pooled in a LowBind 1.5 ml Eppendorf tube and NMM (315 μ L, 0.4M in DMSO, 2100 eq.) was added. The trifunctional linker (120 μ L, 0.5M in DMSO, 1000 eq.) was added, followed by DMTMM-BF₄ (150 μ L, 0.4M in DMSO, 1000 eq.). The solution was shaken at RT for 24 h. The mixture was purified by ethanol precipitation and the pool was dissolved in H₂O (540 μ L) to yield a 130 μ M solution. Success of the coupling was checked by HPLC analysis. DE-1 element NP7 was coupled independently since 2 couplings were necessary to achieve full conversion to the desired product. The procedure was conducted at the same reagent concentrations as with the pooled elements. Element NP7 was pooled with the other 20 elements after successful modification.

3.3 Ester Hydrolysis of the DNA-DE-1 Pool



In a LowBind 1.5 ml Eppendorf tube the DNA-DE-1 pool (130 μ M in H₂O, 564 μ L, 1.0 eq.) was mixed with MeCN (340 μ L) and LiOH * H₂O (100 mM in H₂O, 110 μ L, 150 eq.). The solution was shaken at RT for 2.5 h followed by ethanol precipitation. HPLC showed full conversion of the starting material (peak shift). The pool was dissolved in MOPS buffer (648 μ L) to yield a 100 μ M solution.

3.4 Amino Acid Test Couplings for Usage in the DEML Synthesis







































In a PCR tube XX (74 μ M, MOPS buffer, 2.0 μ L 1.0 eq.), NMM (250 mM, DMSO, 2.0 μ L, 3378 eq.), AAm (500 mM, DMSO, 0.3 μ L, 1014 eq.) and DMTMM-BF₄ (75 mM, DMSO, 2.0 μ L, 1014 eq.) were mixed. The reaction was left standing at RT for 19 h. The mixture was purified by EtOH precipitation. The DNA was dissolved in H₂O (6 μ L) and analyzed by HPLC (2 μ L) and LC-MS (4 μ L). 126 amino acids were tested with these conditions. Low yielding or non-converting building blocks were repeated with EDC/HOAt

conditions (NMM was replaced by DIPEA, DMTMM-BF₄ was replaced by EDC*HCl (150 mM, 1.0 μ L) and HOAt (150 mM, 1.0 eq.)). In the case of XX with n=1 the dehydroalanine sideproduct was found along with the desired products. These eliminated species were included in the conversion and purity yields of the amide coupling reaction.

Table 2. DE-2 amino acids screening results. 126 building blocks were tested for their reactivity in amide coupling. Unless stated otherwise the screening was performed with XX (n = 1). Red coloring indicates the building blocks that were excluded from the final DEML assembly due to insufficient conversion and purity. Cutoff: >85% conversion and \geq 50% purity.

No.	Structure	Calc. Mass [m/z]	Obs. Mass [m/z]	Conversion (Purity)	No.	Structure	Calc. Mass [m/z]	Obs. Mass [m/z]	Conversion (Purity)
AA001		1077.3	1077.3	100% (70%)	AA064		1231.3 1233.3	1231.3 1233.3	100% (70%)
AA002		1077.3	1077.3	100% (92%)	AA065		1119.4	1119.4	100% (76%)
AA003		1077.3	1077.3	100% (66%)	AA066		1119.4	1119.4	100% (71%)
AA004		1111.3	1032.2 ^[a]	100% (0%)	AA067		1105.4	1105.4	100% (80%)
AA005		1091.3	879.3 ^[b] 976.3 ^[c]	100% (0%) 100% (0%) ^[d]	AA068		1192.4	1092.4 ^[g]	94% (69%)
AA006		1159.4	1159.4	100% (75%)	AA069		1192.4	1092.4 ^[g]	100% (66%)
AA007		1154.4	1154.3 1168.3 ^[e]	62% (34%) 100% (89%) ^[d]	AA070		1206.4	1106.4 ^[g]	95% (72%)
AA008		1241.5	1241.4	100% (90%)	AA071		1091.3	879.3 ^[b]	100% (<5%)
AA009		1105.4	1105.3	100% (83%)	AA072		1091.3	1091.4	100% (72%)
AA010		1105.4	1105.3	100% (82%)	AA073		1101.3	1101.4	100% (66%)
AA011		1119.4	1074.3 ^[f]	100% (0%)	AA074		1119.4	1119.3	90% (60%)
AA012		1063.3	1063.3	100% (92%)	AA075		1091.3	1091.4	100% (72%)
AA013		1077.3	1077.3	100% (82%)	AA076		1119.4	1119.4	94% (79%)
AA014		1139.3	1139.3	100% (75%)	AA077		1079.3	879 ^[b] 1024.3 ^[f]	100% (0%)
AA015		1153.4	1153.3	100% (79%)	AA078		1209.4	1209.4	95% (73%)
AA016		1155.3	1155.3	63% (39%) 100% (78%) ^[d]	AA079		1147.4	1147.4 976.3 ^[c]	95% (42%) 100% (32%) ^[d]
AA017		1177.4	1177.3	100% (73%)	AA080		1119.4	1119.4	100% (73%)
AA018		1157.4	1157.3	100% (91%)	AA081		1133.4	1133.4	95% (32%)
AA019		1213.4	1213.3	95% (73%)	AA082		1147.4	1147.4	90% (31%)
AA020		1169.4	1169.3 1183.3 ^[e]	63% (39%) 100% (62%) ^[d]	AA083		1102.3	1031.3 ^[f]	100% (0%)

AA021		1169.4	1169.3	71% (54%) 100% (71%) ^[d]	AA084		1170.3	1170.3	100% (69%)
AA022		1183.4	1138.3	100% (0%)	AA085		1159.3	879.3 ^[b]	100% (0%)
AA023		1421.2	1435.2 ^[e]	100% (35%) 100% (63%) ^[d]	AA086		1087.3	1087.4	93% (72%)
AA024		1134.4	1134.3	100% (89%)	AA087		1089.3	1089.4	100% (69%)
AA025		1093.3	1093.3	100% (86%)	AA088		1089.3	1089.4	100% (75%)
AA026		1093.3	1093.3	100% (89%)	AA089		1129.4	1129.4	100% (75%)
AA027		1119.4	1119.4	100% (72%)	AA090		1117.4	1117.4	97% (54%)
AA028		1133.4	1088.3 ^[f]	100% (0%)	AA091		1131.4	1131.4	92% (78%)
AA029		1192.4	1192.3	100% (71%)	AA092		1131.4	1131.4	88% (65%)
AA030		1192.4	1192.3	100% (78%)	AA093		1103.3	1103.4 1117.4 ^[e]	100% (45%) 100% (70%) ^[d]
AA031		1206.4	879.3 ^[b]	100% (<5%)	AA094		1089.3	1089.3	36% (36%) 100% (86%) ^[d]
AA032		1107.3	1107.3	100% (88%)	AA095		1159.4	879.3 ^[b]	100% (0%)
AA033		1107.3	1107.3	100% (90%)	AA096		1103.3	1103.4	90% (37%) 100% (24%) ^[d]
AA034		1107.3	1107.3	100% (90%)	AA097		1143.4	1143.3	98% (58%)
AA035		1107.3	1107.3	100% (90%)	AA098		1145.4 1033.3 ^[f]		100% (<5%)
AA036		1207.4	1207.4	100% (86%)	AA099		1193.4	1193.3	100% (71%)
AA037		1207.4	1207.4	100% (89%)	AA100		1181.3	949.3 ^[a]	56% (0%)
AA038		1220.4	1120.4 ^[g]	94% (74%)	AA101		1225.5	1225.4	95% (85%)
AA039		1119.4	1119.3	100% (72%)	AA102		1143.4	1143.4	100% (60%)
AA040		1119.4	1119.4	100% (71%)	AA103		1159.4	1159.4	89% (43%)
AA041		1234.4	1134.4 ^[g]	98% (75%)	AA104		1148.3	910.2 ^[f]	95% (0%)

AA042		1268.4	1268.4	100% (85%)	AA105		1167.3	879.3 ^[b]	100% (0%)
AA043		1234.4	1134.4 ^[g]	23% (13%)	AA106		1240.4	1240.4	93% (76%)
AA044		1137.3	1137.3	100% (76%)	AA107		1160.3	1160.3	93% (66%)
AA045		1137.3	1137.3	100% (78%)	AA108		1204.4	1204.4	100% (71%)
AA046		1191.4	1191.4 ^[h]	100% (73%)	AA109		1251.4	1251.4	100% (73%)
AA047		1120.3	1120.3	100% (80%)	AA110		1203.4	1203.4	87% (71%)
AA048		1103.3	1103.3	95% (72%)	AA111		1167.4	1167.4	100% (63%)
AA049		1103.3	1103.3	100% (82%)	AA112		1167.4	1167.4	95% (52%)
AA050		1117.4	1117.3 879.3 ^[b] 976.3 ^[c]	100% (<5%) 100% (0%) ^[d]	AA113		1185.4	1185.4	95% (60%)
AA051		1117.4	1117.3	100% (<5%)	AA114		1179.4	1179.4	100% (50%)
AA052		1119.3	1119.3	100% (75%)	AA115		1197.4	1197.4	95% (73%)
AA053		1119.3	1119.3	100% (83%)	AA116		1181.4	1181.4	93% (51%)
AA054		1101.3	1101.3	100% (82%)	AA117		1199.4	1199.4	100% (60%)
AA055		1153.4	1153.3	100% (75%)	AA118		1187.3	1187.4	93% (50%)
AA056		1153.4	1153.3	100% (78%)	AA119		1229.4	1229.4	94% (58%)
AA057		1167.4	1167.3 879.3 ^[b] 976.3 ^[c]	100% (<5%) 100% (0%) ^[d]	AA120		1217.4	1217.4	94% (65%)
AA058		1198.3	1198.3	100% (74%)	AA121		1145.3	1145.3	95% (64%)
AA059		1198.3	1198.3	100% (57%)	AA122		1143.4	1143.3	100% (60%)

AA060		1167.4	879.3 ^[b] 1122.3 ^[f]	100% (<5%)	AA123		1247.4	1247.4	100% (76%)
AA061		1185.4	1185.4	100% (79%)	AA124		1143.4	1143.4	100% (74%)
AA062		1171.4	1171.4	100% (76%)	AA125		1129.4 ^[e]	1129.3	100% (69%)
AA063		1171.4	1171.4	100% (76%)	AA126		1174.3 ^[e]	1174.3	100% (77%)

[a] Elimination reaction(s) to dehydroalanine species **XA**. [b] Terminal amide sideproduct **XB**. [c] Dihydrotriazinone **XC** [d] EDC/HOAt/DIPEA reaction conditions. [e] Trifunctional linker with n=2 [f] unknown species [g] Boc removal at ESI-MS conditions [h] tBu ester hydrolysis in ESI-MS also observed

From **Table 2** we found trends in reactivity for amide coupling depending on the amino acid structure. Generally, *N*-methylated amino acids did not couple (except AA013, sarcosine) as well as α , α - disubstituted amino acids (AA093 in combination with the EDC/HOAt/DIPEA system was the exception). Cyclic amino acids also showed a reactivity trend depending on the nature of the ring scaffold. The 4-membered ring amino acid (AA094) as well as the proline derivatives (AA048, AA049, AA052-AA054) worked well under the tested reaction conditions whereas homoprolines (AA050, AA051) did not work at all. Interestingly, β - and γ - homoprolines (AA091, AA092, AA099) yielded a good amount of the desired product, which leads to the conclusion that the arrangement of the functional groups in cyclic amino acids has a big influence on their reactivity. A very important but difficult group of compounds were linear β - amino acids. In the case of α - substituted β - amino acids (AA080, AA101, AA120) the couplings proceeded with good conversions and purities whereas with β - substituted β - amino acids only the members with flat, aromatic sidechains (AA111 - AA113, AA115 - AA119) gave good enough yields. The special case amino acids with electron - withdrawing substituents (AA083, AA085), good leaving groups (AA004), a hydroxylamine (AA077) and a thiazoline ring scaffold (AA104) generally showed no conversion to the desired products at all. Due to the reactivity of aromatic alcohols with DMTMM, tyrosine derivatives were only successfully coupled under EDC/HOAt/DIPEA conditions. We could identify by LC-MS the two major sideproducts that we observed during the test assay.

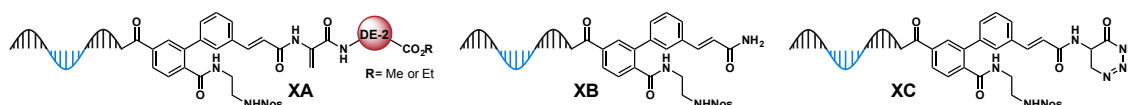
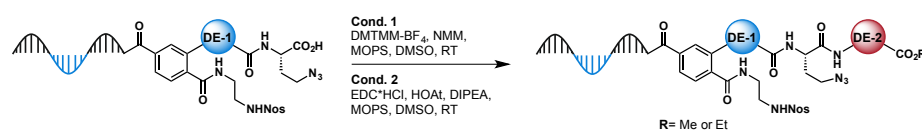


Figure 9 Proposed structure of found common sideproducts. Elimination product to the dehydroalanine species **XA**, terminal amide product **XB** and dihydrotriazinone product **XC**.

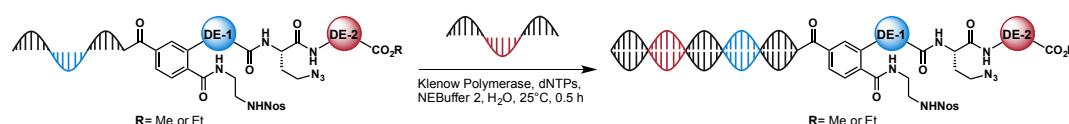
3.5 Amino Acid Couplings, DE-2 Attachment by Split Synthesis



The amide coupling reactions were performed in U-shaped 96 well-plates (Eppendorf Microplate 96/U-PP, white border, PCR clean, 250 μ L well-volume). The stated reagent amounts apply per well.

The DNA-DE-1 pool (90 μ M in MOPS buffer, 6.5 μ L, 1.0 eq.) was distributed in 102 wells on 2 plates. NMM (250 mM in DMSO, 8.2 μ L, 3500 eq.) was added, followed by the 102 amino acids (500 mM in DMSO, 1.2 μ L, 1000 eq.). DMTMM-BF₄ (150 mM in DMSO, 3.9 μ L, 1000 eq.) was added and the solutions were thoroughly mixed. The plates were slowly shaken at RT for 20 h. NaOAc buffer (3M, pH 5.20, 2.5 μ L) was added, followed by EtOH (75 μ L). The plates were placed in the fridge overnight. The plates were centrifuged (3700 rpm, 4°C, 60 min) and the supernatant was discarded. The DNA pellets were washed with EtOH (75%, 2 x 50 μ L) and the plates were centrifuged (3700 rpm, 4°C, 2 x 40 min). The purified pellets were dried in the air for 20 min. The DNA pellets were dissolved in H₂O (20 μ L) to yield 18-20 μ M solutions (average of 9 measurements). For Amino Acids AA007, AA016, AA020, AA021, AA023, AA093 and AA094 NMM was replaced by DIPEA (250 mM in DMSO, 8.2 μ L, 3500 eq.) and the coupling reagents were changed to EDC·HCl (300 mM in DMSO, 2 μ L, 1000 eq.) and HOAt (300 mM in DMSO, 2 μ L, 1000 eq.).

3.6 Klenow Encoding of the Coupled DE-2 Building Blocks

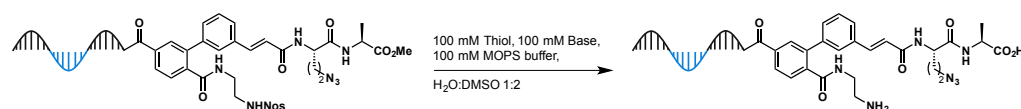


The encodings were performed in PCR tube 96 well-plates (Eppendorf twin.tec PCR Plate 96 LoBind, semi-skirted, 250 μ L well-volume). The amino acid coupling products (20 μ M in H₂O, 20 μ L) were transferred to the PCR 96 well-plates and diluted with H₂O (146 μ L) and 10 X NEBuffer 2 (20 μ L). The DE-2-encoding DNA strand (100 μ M in H₂O, 8 μ L, 2.0 eq.) with the general sequence 5'-GTA GTT GGA TCC GCA CAC YYYYY GAC AAT TCA CAC ACG TCC-3' was added and the solution was annealed by heating to 65°C for 5 min. YYYYY represents the coding sequence for the DE-2 amino acids and the underline shows the BamHI restriction site. The used annealing gradient was: 65°C(5)-50°C(0.5)-40°C(0.5)-25°C. The dNTPs mixture (10 mM in H₂O each dNTP, 4 μ L, 100 eq.) was added, followed by the Klenow polymerase (5000 U/ml, 2 μ L, 10 Units). The reaction was incubated at 25°C for 30 min while shaking (150 rpm). To stop the reaction, EDTA (500 mM, 5 μ L) was added and the mixture was incubated at 75°C for 20 min. The 102 encoded wells were combined in Eppendorf tubes (2 ml tubes, 14 pcs) and concentrated to 8 ml by SpeedVac (45°C, 6 h). NaOAc buffer (3M, pH 5.2, 960 μ L) was added, followed by EtOH (28.8 mL). The mixture was left in the fridge (4°C) overnight. The suspension was centrifuged (4200 rpm, 4°C, 60 min) and the supernatant was discarded. The pellet was resuspended in EtOH (75%, 2 x 8 ml) and centrifuged (4200 rpm, 2 x 30 min, 4°C). The clean pellet was dried in air for 20 min. The DNA was dissolved in MOPS buffer (300 mM, 500 mM NaCl, pH 8.2, 800 μ L) to yield a 200 μ M solution. Success of the encoding was checked by native DNA polyacrylamide gel electrophoresis (12%, TBE, 150 V, 75 min, SYBR Gold staining).



Figure 10. Left: Scheme for the encoding of the 2nd diversity element by Klenow extension. Right: 12% DNA polyacrylamide gel, SYBR gold staining. L1: Low MW DNA ladder, L2: DNA-DE-1 pool, L3: DE-2 coding DNA strand, L4: Annealing of DNA-DE-1 pool and DE-2 coding DNA strand, L5: Klenow extension.

3.7 Optimization of the Ester and NOSYL Deprotection



Stock solutions: DNA test sample (NP1/AA001, 87 μ M in 300 mM MOPS 500 mM NaCl buffer, 2.0 μ L, 1.0 eq.), thiol (300 mM in DMSO, 2.0 μ L, 3448 eq.) base (300 mM in DMSO, 2.0 μ L, 3448 eq.).

In a PCR tube all stock solutions were mixed and the solution was degassed with N₂ for 30 s, followed by incubation according to **Table 3**. The sample was purified by ethanol precipitation, dissolved in H₂O (6 μ L) and further analyzed by HPLC and LC-MS.

Table 3. NOSYL deprotection screening and optimization.

Entry	Thiol	Base	Temperature	Time	NOSYL Deprotection	Ester Hydrolysis
1	PhSH	DIPEA	60°C	6 h	Yes	No
2	BME	DBU	60°C	6 h	Yes	Yes
3	MPAA	DIPEA	60°C	6 h	Incomplete	No
4	NaTG ^[a]	DIPEA	60°C	6 h	No	No
5	PhSH	DBU	60°C	2 h	Incomplete	Incomplete
6	PhSH	DBU	60°C	4 h	Yes	Yes
7	PhSH	DBU	60°C	6 h	Yes	Yes
8	BME	DBU	RT	2 h	Yes	Yes
9	BME	DBU	RT	4 h	Yes	Yes
10	BME	DBU	RT	6 h	Yes	Yes
11 ^[b]	MPAA	DBU	60°C	6 h	Incomplete	Incomplete

12	BME	DIPEA	RT	6 h	Incomplete	No
13	BME	NMM	RT	6 h	No	No

[a] Stock solution 1:1 H₂O:DMSO. [b] Reagent concentrations: 200 mM.

We analyzed the influence of the sulfur nucleophile in combination with different bases at variable temperatures on product and sideproduct (mainly terminal amide **XB**) formations. Only thiophenol (PhSH) and β - mercaptoethanol (BME) completely removed the NOSYL group. Ester deprotection was uniquely achieved in combination with DBU. The mildest conditions with the least sideproduct formation were BME/DBU at RT (**Table 3**, Entry 10). These findings were well in accordance to a published procedure on NOSYL deprotection with DNA-encoded amino acids on a solid support from Halpin et al.^[4] Ester hydrolysis (with LiOH) prior to NOSYL removal was very inefficient and led to a high amount of the undesired sideproduct. We further investigated the influence of the diversity element 2 structure on deprotection efficiencies. For this purpose we synthesized a series of encoded macrocycle precursors with constant DE-1 (NP19) and 20 diverse DE-2 elements (**Figure 11**). The best protecting group removal was achieved with proline derivatives (AA049, AA053 and AA106), but also the common amino acids Gly, Val and Phe (AA012, AA009, AA010, AA056, AA058) showed good deprotection. The size and bulkiness of the amino acids seemed to have a big influence. The sterically hindered dicyclohexylalanine (AA008) and the adamantyl amino acid (AA101) showed little to no conversion but the formation of a substantial amount of sideproduct. Despite good conversions to the desired products, sarcosine (AA013), methylhistidine (AA018) and serine (AA025) were amongst the members with the highest sideproduct formation of all tested compounds. *L*-Asparagine (AA047) with its nucleophilic amide sidechain underwent an intramolecular cyclization reaction with the adjacent ester to form the corresponding cyclic imide **XD**. From these findings especially the building blocks AA008, AA047 and AA101 should be excluded from the library assembly in a future modified resynthesis.

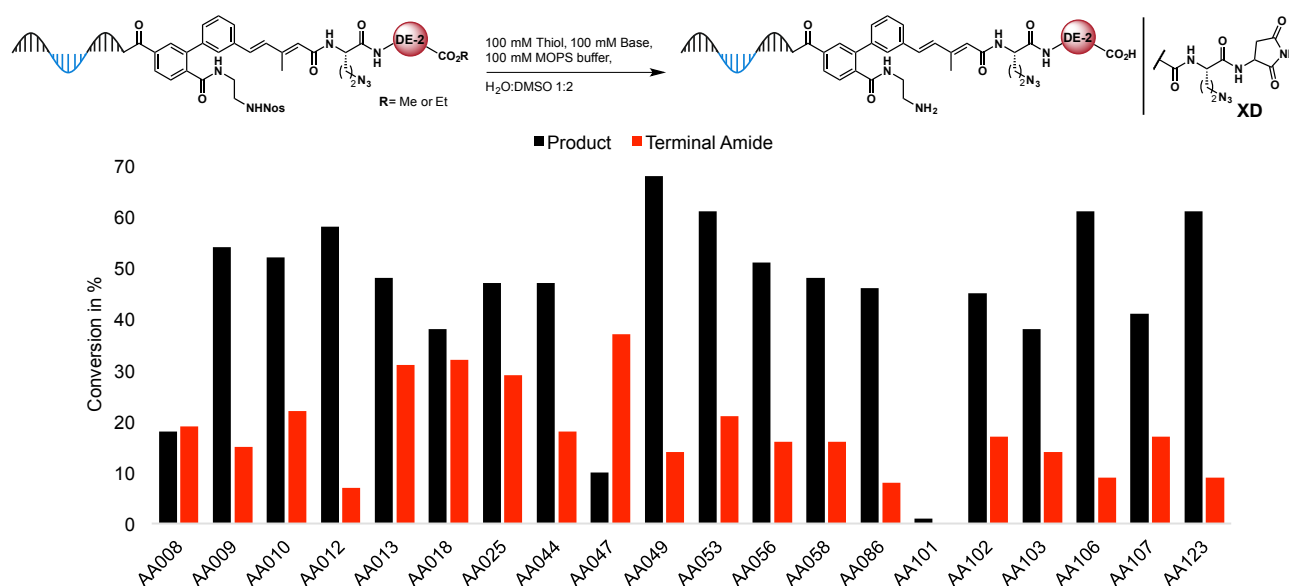
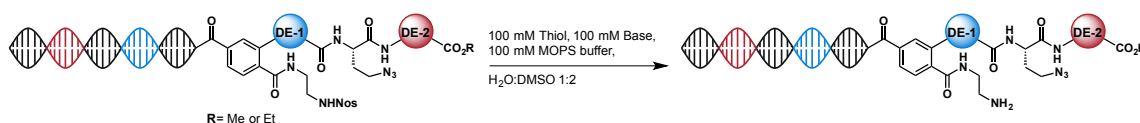


Figure 11. Nosyl deprotection results with 20 representative DE-2 building blocks, including the most special amino acids in terms of size and structure. The desired products are shown with black pillars, the undesired terminal amide sideproduct **XB** is represented in red.

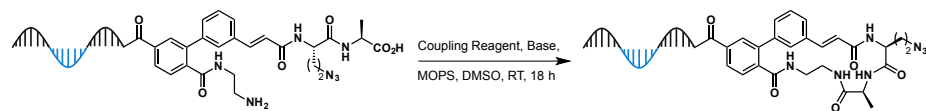
3.8 Ester and NOSYL Deprotection



In a 5 ml Eppendorf tube the encoded library (800 μ L in 300 mM MOPS, 500 mM NaCl buffer), DBU (300 mM in DMSO, 800 μ L) and BME (300 mM in DMSO, 800 μ L) were mixed. The mixture was degassed with N₂ for 30 s, followed by shaking at RT for 6 h. A white precipitate formed after BME addition. The suspension was treated with NaOAc buffer (3M, pH 5.2, 250 μ L) and EtOH (8.1 ml) and was placed in the fridge (4°C) overnight. The mixture was centrifuged (4200 rpm, 4°C, 60 min) and the supernatant was discarded.

The pellet was washed with EtOH (75%, 2 x 1 ml) and centrifuged (4200 rpm, 4°C, 2 x 30 min), then dried in the air for 30 min. The DEML was dissolved in MOPS buffer (700 µL) to yield a 79 µM solution. Success of the transformation was checked by HPLC (peak shift).

3.9 Macrocyclization Optimization



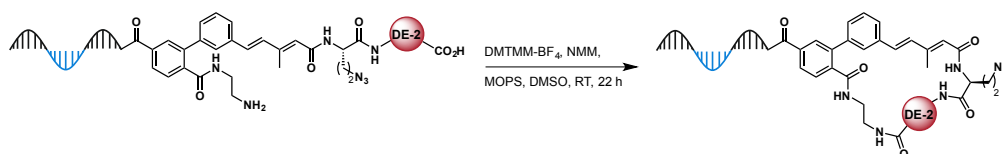
In a PCR tube the DNA sample (**NP1/AA001**, 30 µM in MOPS buffer, 3.0 µL) was mixed with DMSO (2.4 µL), base (1.8 µL in DMSO) and coupling reagent (1.8 µL in DMSO). The reaction was left standing at RT for 18 h. The reaction was purified by ethanol precipitation and the DNA pellet was dissolved in H₂O (5 µL) and analyzed by LC-MS.

Table 4. Efficiency of the macrocyclization with different coupling reagents and amounts.

Entry	Coupling Reagent	Equivalents	Base	Macrocyclization
1	DMTMM-BF ₄	500	NMM	Yes
2	EEDQ	500	-	No
3	EDC ⁺ HCl/sulfo-NHS	500	DIPEA	very little
4	DMTMM-BF ₄	250	NMM	Yes
5 ^[a]	DMTMM-BF ₄	100	NMM	Yes

[a] Bigger amount of sideproduct formation.

We tested the influence of different coupling reagents on the efficiency of the macrocyclization reaction. DMTMM-BF₄ clearly showed to be superior compared to the other used reagents (see **Table 4**). Unlike for common amino acid couplings, the macrocyclizations could be performed with fewer equivalents of the reagents (250 - 500 eq.). Lowering the amount of coupling reagent to as low as 100 eq. the cyclization reaction still occurred within the tested time frame, but a higher amount of sideproducts was found. We think that this results from the slower kinetics of the cyclization reaction, which then increases the sideproduct formation. Optimal conditions were found with 250 - 500 eq. of coupling reagent and 1000 eq. of base. The DNA concentration in the final mixture was 10 µM to avoid dimerization products. Even concentrations down to 1 µM still showed very efficient couplings. We further investigated on the influence of the DE-2 elements for macrocyclization reactions. As shown in **Figure 12**, the majority of the tested compounds showed cyclization ratios of >60%. Only amino acids that also caused difficulties during the preceding NOSYL deprotection (AA008, AA047 and AA101) were not cyclized with this method. For AA008 and AA101 the bulky sidechains had an influence on the deprotection reaction by which only a very small amount or even no unprotected species was formed. Moreover, the bulky groups of these members potentially inhibited the activation of the carboxylic acid and/or prevented the amine from the nucleophilic attack. **NP19/AA047** was not formed because the asparagine moiety was completely transformed to the imide sideproduct **XD** (**Figure 11**).



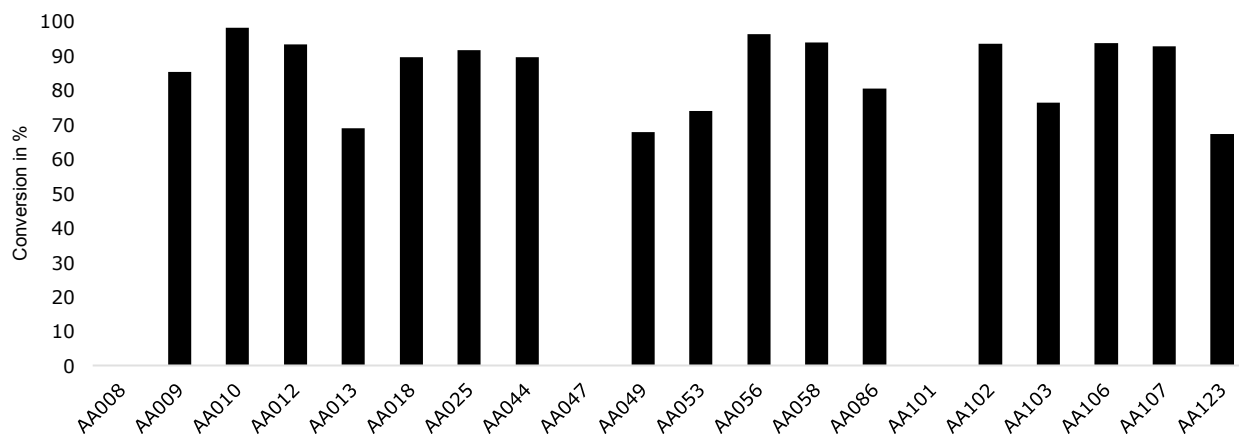
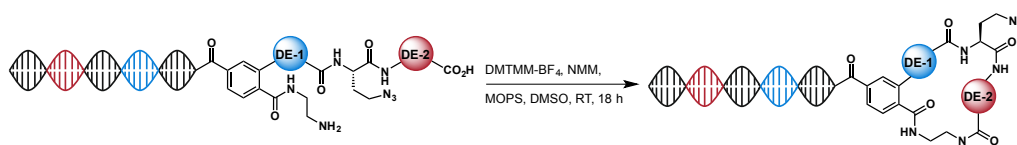


Figure 12. Macrocyclization efficiencies depending on the DE-2 building block.

3.10 Macrocyclization of the Encoded DE-1/DE-2 Pool



In a 5 ml Eppendorf tube the DEML (900 μ L, 56 μ M, in MOPS buffer) was diluted with MOPS buffer (1.35 ml) and DMSO (637 μ L). NMM (50 mM in DMSO, 1.008 mL, 1000 eq.) and DMTMM-BF₄ (25 mM in DMSO, 605 μ L, 300 eq.) were mixed and shaken at RT for 18 h. NaOAc buffer (3M, pH 5.2, 450 μ L) was added, followed by EtOH (15 ml). The suspension was placed on ice for 2 h, followed by centrifugation (4200 rpm, 4°C, 60 min) and the supernatant was discarded. The DNA pellet was washed with EtOH (75%, 2 x 4 ml), centrifuged (4200 rpm, 4°C, 2 x 30 min) and dried in the air for 30 min. The DEML pellet was dissolved in H₂O (800 μ L). The coupling was repeated once again.

The DEML was purified by ssDNA digestion in 8 batches, followed by RP preparative Chromatography. The library (100 μ L) was diluted with nuclease-free H₂O (770 μ L) and Exonuclease 1 buffer (100 μ L) was added, followed by the Exonuclease 1 enzyme (20000 U/ml, 30 μ L, 600 U). The solution was incubated at 37°C for 40 min and the enzyme was deactivated by heating to 80°C for 20 min. The crude material was directly purified by prep HPLC (Method B). Product containing fractions were combined, lyophilized and dissolved in nuclease-free H₂O (1.2 ml, 26 μ M). 100 μ L of the DEML solution was diluted with TRIS (10 mM, pH 8.0) to a 1 μ M final concentration. The library was aliquoted (10 μ L aliquots) and stored in the freezer (-20°C) for protein selection experiments (small DEML).

Note: The macrocyclization was repeated once because the reference sample, which was synthesized simultaneously showed incomplete conversion. Increase of the coupling reagent amount (up to 500 eq.) and DMSO amount (>55%) should yield full macrocyclization in one step.

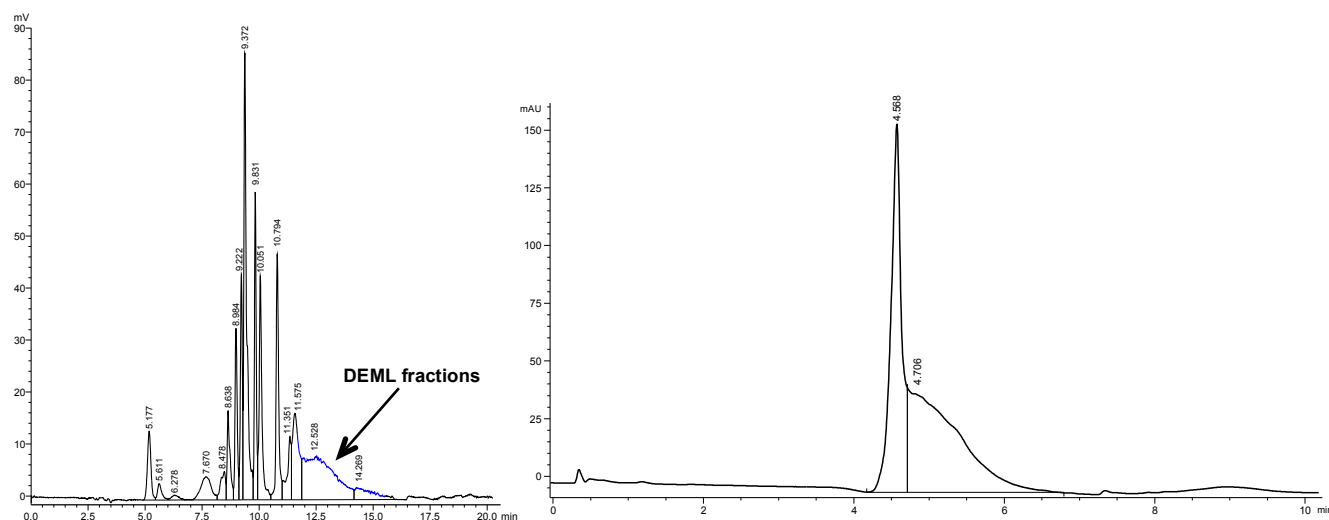
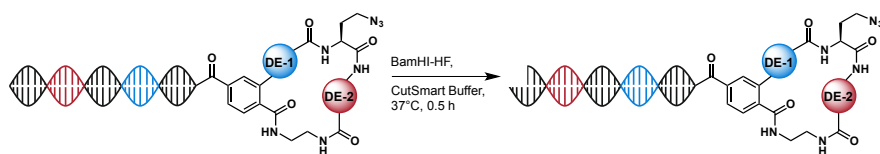


Figure 13. Left: Preparative HPLC chromatogram at 254 nm for the purification of the DEML. Blue indication shows the DEML containing fractions. Right: HPLC chromatogram at 254 nm of the purified small DEML. The large peak at 4.568 min contained some residual coding DE-2 coding DNA strands from the last encoding step.

3.11 BamHI-HF Restriction Digest



The restriction digest of the DEML was performed in 20 batches; amounts per batch.

In an Eppendorf tube, DEML (55 μ L, 26 μ M in H₂O) was mixed with 10X CutSmart buffer (75 μ L) and diluted with H₂O (565 μ L). BamHI-HF (55 μ L, 1100 U, 20000 U/mL) was added and incubated at 37°C for 30 min. The success of the reaction was checked by native polyacrylamide gel electrophoresis (12%, TBE, 150 V, 70 min). The reaction was purified by phenol-chloroform extraction. A solution of phenol:chloroform:isoamylalcohol 24:24:1 (740 μ L) was added to the reaction and the mixture was vortexed for 2 min. The biphasic mixture was centrifuged (16900 g, RT, 5 min) and the aqueous layer was carefully removed. The aqueous phase was washed with chloroform (740 μ L), vortexed for 2 min and centrifuged (16900 g, RT, 5 min). The aqueous layer was carefully removed and concentrated in SpeedVac (45°C, 4 h). The 20 combined samples were diluted with H₂O to 4 mL total volume. NaOAc buffer (3 M, pH 5.2, 0.4 mL) was added, followed by EtOH (13.2 mL). The mixture was placed on ice for 1 h and in the freezer at -80°C for 1 h. The suspension was centrifuged (4200 rpm, 4°C, 1 h) and the supernatant was removed. The pellet was washed with EtOH (75%, 2 x 4 mL) and centrifuged (4200 rpm, 4°C, 2 x 20 min). The restricted DNA was dried in the air for 0.5 h. The DNA pellet was dissolved in TEAA buffer (0.5 M, pH 7.2, 2345 μ L) to yield a 10.5 μ M solution.

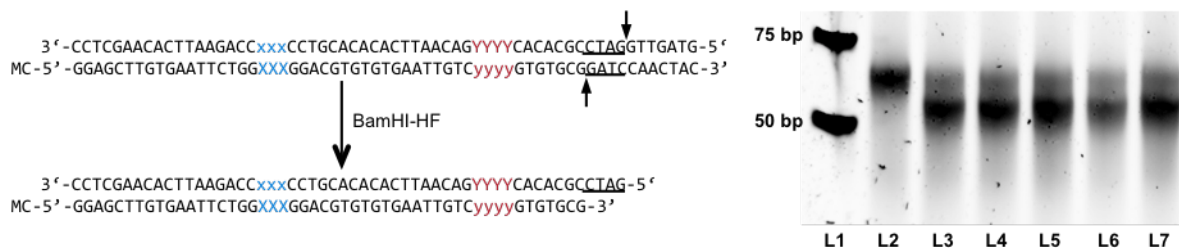
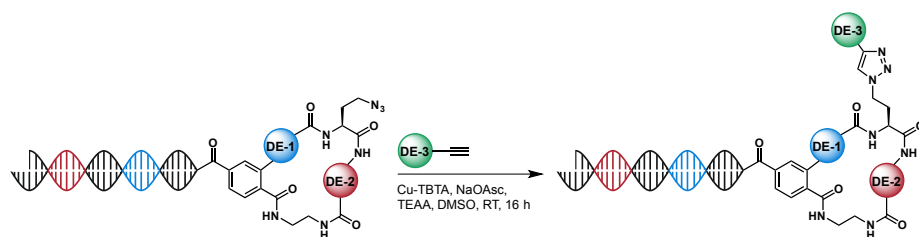


Figure 14. Left: Restriction scheme. Underlined bases indicate the recognition site for the BamHI-HF enzyme, the arrows show the restriction sites. Right: Native PAGE gel of several restriction samples. L1: Low MW DNA ladder, L2: small DEML before restriction, L3-L7: Restricted samples.

For the encoding of the third diversity element, the coding oligonucleotide was restricted with BamHI-HF to create a four bases 5' overhang (sticky end). The restriction process is shown in **Figure 14** on the right. The enzyme did not completely restrict the DNA, about 20% uncut DNA was still found. Any condition changes (amount of enzyme, duration, double restriction) did not improve the restriction rate of the enzyme. The restricted library was used as obtained for further experiments.

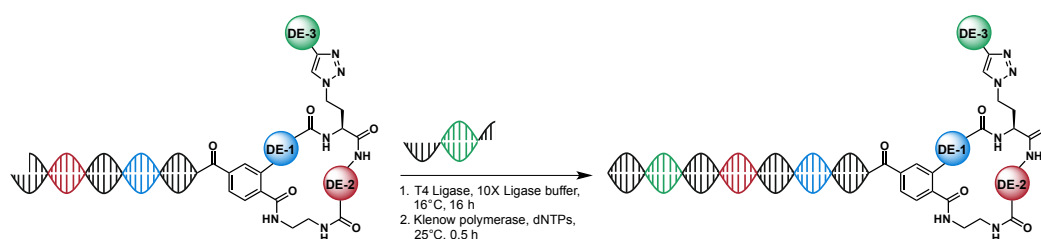
3.12 Click Reactions, DE-3 Incorporation by Split Synthesis



The reactions were performed in U-shaped 96 well-plates (Eppendorf Microplate 96/U-PP, white border, PCR clean, 250 μ L well-volume). Columns 1 and 12 were left empty, 80 reactions per plate. 663 reactions were performed in 9 plates. The stated reagent amounts are given per well. Addition of the catalyst and the reducing agent was performed in a glove bag under an inert atmosphere (N₂).

The restricted DEML (3.5 μ L, 10.5 μ M in 0.5 M TEAA buffer, pH 7.2) was mixed with the DE-3 alkyne (3.7 μ L, 1 mM in DMSO, 100 eq.) and DMSO (2.5 μ L). Under an inert atmosphere degassed NaOAsc solution (2.45 μ L, 3 mM in H₂O, 200 eq.) was added, followed by Cu(II)-TBTA complex solution (2.45 μ L, 3 mM in 55% DMSO/H₂O, 200 eq.). The plates were sealed (Starlab StarSeal sealing tape polyolefin) and left standing in the glove bag at RT for 16 h. NaOAc buffer (3M, pH 5.2, 2.5 μ L) was added, followed by EtOH (65 μ L). The plates were placed in the fridge over the weekend. The 96-well plates were centrifuged (3700 rpm, 4°C, 60 min) and the supernatant was removed. The DNA was washed with EtOH (75%, 2 x 50 μ L) and centrifuged (3700 rpm, 4°C, 2 x 30 min). The DNA pellet was dissolved in nuclease-free H₂O (10 μ L) for the following encoding reaction.

3.13 Encoding of DE-3 by T4 Ligation and Klenow Fill-in



The encoding was performed in PCR tube 96 well-plates (Eppendorf twin.tec PCR Plate 96 LoBind, semi-skirted, 250 μ L well-volume). The given amounts and volumes are valid per well. DE-3 encoding DNA strands (called insert DNA) consisted of a partially double stranded DNA piece, that was preformed by the annealing of the coding strand with the sequence 5'-GTT CAA GCC ACT TAC CTZ ZZZ ZZZT GAT GCC TAC CTA TGA GA-3' and a 5' phosphorylated strand with the sequence 5'-P-GAT CCA AGT TCG GTG AAT GGA-3'. ZZZ ZZZ stands for the coding sequence of the DE-3 building blocks.

The DEML (10 μ L, approx. 3.7 μ M) was diluted with nuclease-free H_2O (15.3 μ L) and mixed with the insert DNA (2.22 μ L, 50 μ M in H_2O , 3 eq.). 10X ligase buffer (3.5 μ L) and T4 DNA ligase (4 μ L, 80 U, 20000 U/mL) were added. The 96-well plates were incubated at 16°C for 16 h. The reaction was stopped by heating to 65°C for 10 min. dNTPs solution (3.7 μ L, 1 mM in H_2O , 100 eq.) was added followed by Klenow polymerase (4 μ L, 2 U, 500 U/mL) and incubated at 25°C for 30 min. The reaction was stopped by the addition of EDTA (125 mM in H_2O , 4 μ L) and heating to 75°C for 20 min. All 663 reactions were pooled in 2.0 mL Eppendorf tubes and concentrated in the SpeedVac at 60°C. The concentrates were mixed with NaOAc buffer (3M, pH 5.2, 840 μ L) was added followed by EtOH (28 mL). The mixture was put on ice for 3 h and kept in the fridge overnight. The suspension was centrifuged (4200 rpm, 4°C, 60 min) and the supernatant was discarded. The precipitate was washed with EtOH (75%, 2 x 8 mL) and centrifuged (4200 rpm, 4°C, 2 x 20 min). The DNA pellet was dried in the air for 1 h and dissolved in H_2O (1 mL). The encoded macrocycle library was purified by semi-preparative HPLC (Method C). The product-containing fractions were combined, lyophilized and dissolved in 10 mM TRIS buffer pH 7.42 to a final concentration of 9.4 μ M (3890 μ L). The library was aliquoted (10 μ L) and stored in the freezer (-20°C).

Figure 15a shows the encoding scheme for the last diversity element. After the encoding, the DEML was purified by semi-preparative HPLC (**Figure 15b**) to remove enzyme, buffer and excess DNA leftovers. The purification step was not completely successful since we still see bands for short DNA fragments in the final PAGE analysis (**Figure 15c**). Even though we considered the library useful without further purification since the HPLC chromatogram (**Figure 15d**) showed no potential purification improvement by the applied methods. The short DNA strands should not contain any attached small molecules and were not coding for the complete library. These excess oligonucleotides were finally washed away during protein selections.

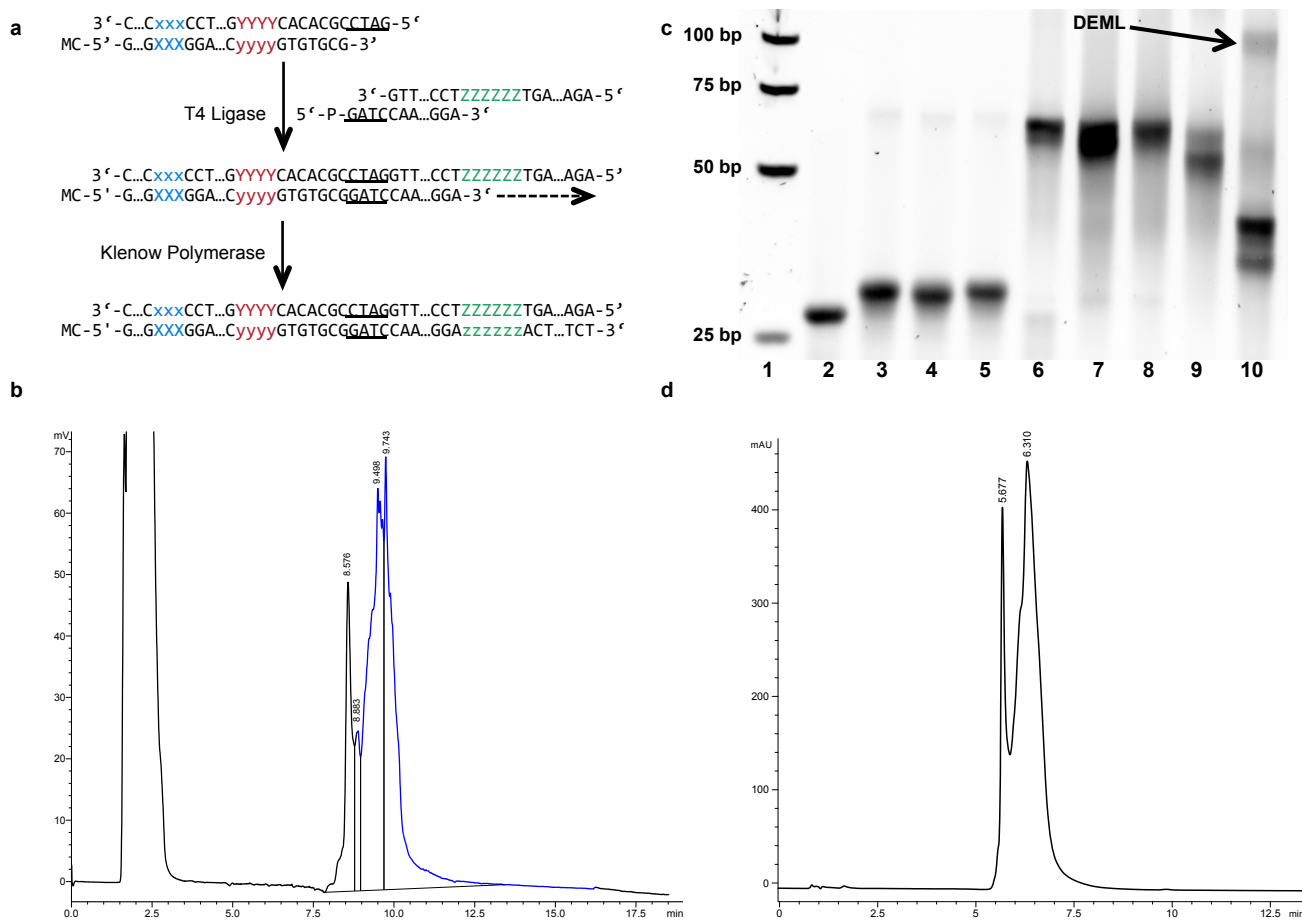


Figure 15. a) Encoding scheme of the 3rd diversity element. Coding regions are colored according to the diversity elements. Underlined bases show the BamHI-HF binding site. **b)** DEML purification on the semi-preparative column. Blue indication shows the library containing fractions. **c)** Full synthesis native DNA polyacrylamide gel (10%, TBE). L1: Low MW DNA ladder, L2: DNA alkyne, L3: DE-1 click reaction, L4: Trifunctional linker coupling, L5: Trifunctional linker ester hydrolysis, L6: DE-2 amide coupling and Klenow encoding, L7: Ester hydrolysis + NOSYL deprotection, L8: Macrocyclization (small DEML), L9: BamHI-HF restriction digest, L10: Click reaction with DE-3 and subsequent encoding by T4 ligation and Klenow fill-in. **d)** HPLC analysis of the purified final library. DEML and left-over DNA strands from the encoding steps overlap.

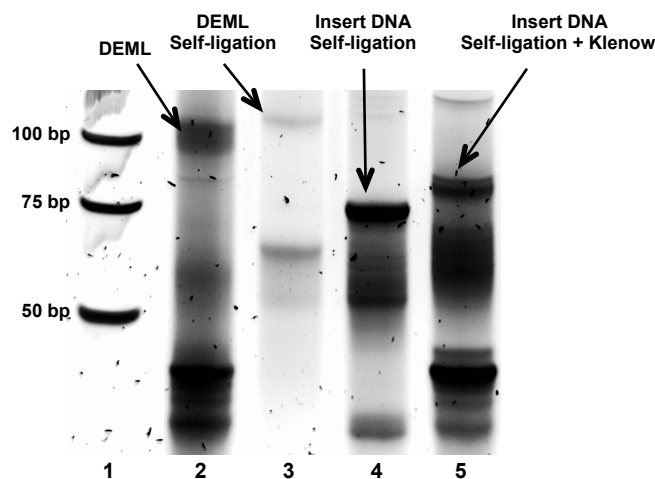


Figure 16. The purified DEML in comparison with self-ligated library or insert DNA strands. L1: Low MW DNA ladder, L2: Purified DEML, L3: DEML self-ligation assay, L4: Insert DNA self-ligation assay, L5: Insert DNA self-ligation assay after Klenow extension.

The palindromic nature of the BamHI-HF binding and restriction site made it possible for the restricted oligonucleotides with "sticky" ends to undergo ligation reactions with each other or with itself (self-ligation). The self-ligation of the DEML was particularly critical because in that case the last diversity element was not correctly encoded for and both DNA strands of the duplex would carry the macrocycle. To overcome this issue we used an excess of insert DNA to prevent DEML self-ligation. Comparison of lanes 2 and 3 in **Figure 16** led to the conclusion that DEML self-ligation did not occur during DE-3 encoding. On the other hand the insert DNA formed the self-ligation product in good amount due to its excess in the mixture. Lanes 4 and 5 show the behavior of this excess during the DEML encoding steps. Unexpectedly, the Klenow fragment seemed to truncate the self-ligated DNA in some part rather than just turn

it into a full duplex DNA. These truncated short oligonucleotides were the main DNA impurity in our library, which we could not completely remove.

4. Physicochemical Properties Analysis of the Macrocycle Library

General information

We developed an in-house software to construct the full library from its fragments ([see detailed lists page....](#)) and subsequently calculate chemical properties with software available for free. Our software suite is available on <https://github.com/Gillingham-Lab/DECL-Gen> and has been developed in Python 3.6 with the following packages: Biopython, RDKit, numpy, scipy, and pandas.

Chemical property calculations

The following properties were calculated with RDKit for the macrocycle library: molecular weight, quantitative estimate of drug-likeness, TPSA, aLogP, number of Hydrogen bond donors, number of Hydrogen bond acceptors, number of heteroatoms, number of rotatable bonds, number of N and O, number of NH and OH, number of rings, size of the biggest ring, fraction of carbons that are sp^3 and number of heavy atoms. We compared these calculated results with the guidelines from Whitty^[5] and Kihlberg^[6], which gave us an idea about the (oral) bioavailability of our potential macrocycles.

Table 5. Comparison of the proposed guidelines from Whitty, Kihlberg and Lipinski. MW = molecular weight, AlogP = atom based partition coefficient (logP) calculation, TPSA = topological polar surface area, HBDs = hydrogen bond donors, HBAs = hydrogen bond acceptors, RotBs = rotatable bonds.

	MW [g/mol]	AlogP	TPSA [\AA^2]	No. HBDs	No. HBAs	No. RotBs
Whitty^[a]	$600 \leq x \leq 1200$	$-2 \leq x \leq 6$	$180 \leq x \leq 320$	≤ 12	$12 \leq x \leq 16$	≤ 15
Kihlberg	≤ 1000	$-2 \leq x \leq 10$	≤ 250	≤ 6	≤ 15	≤ 20
Lipinski	≤ 500	≤ 5	$\leq 140^{[b]}$	≤ 5	≤ 10	$\leq 10^{[b]}$

[a] Proposed physicochemical guidelines for oral macrocyclic drugs. [b] From the enhanced rules for conventional small molecule drugs by Veber.^[7]

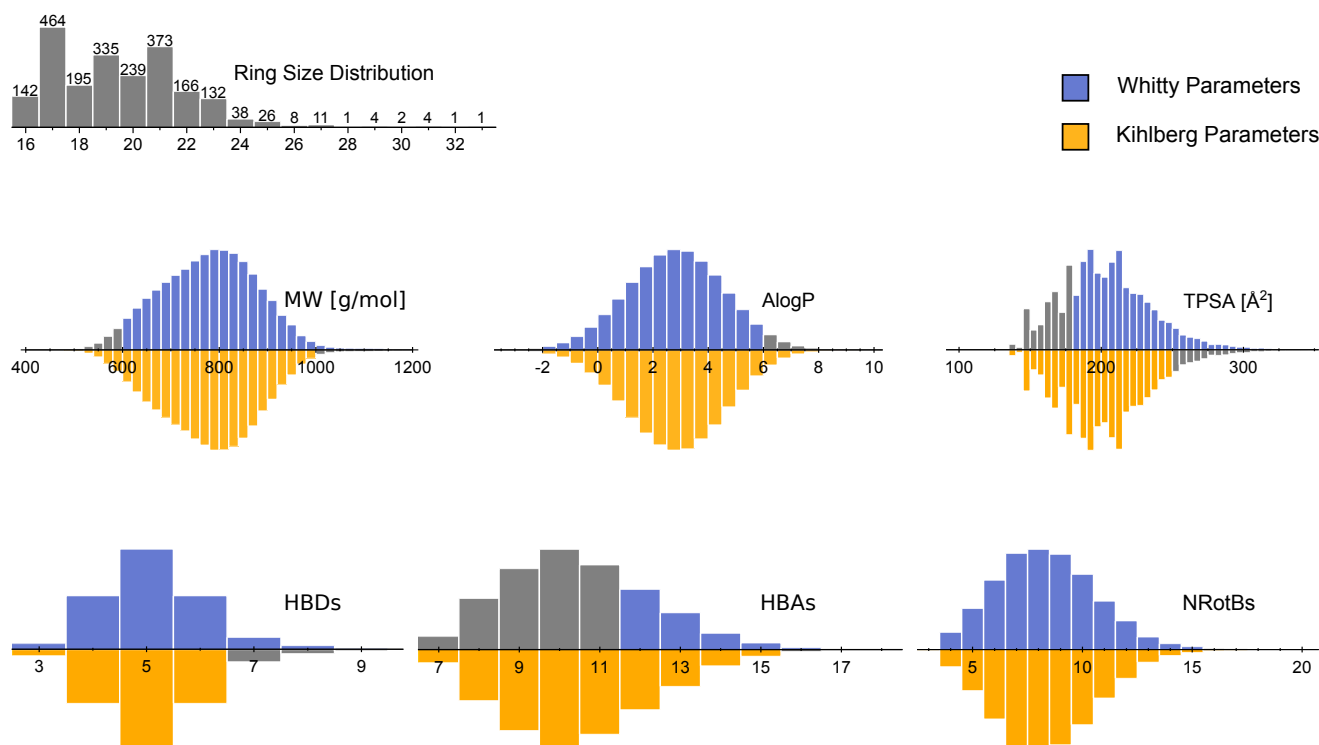


Figure 17. Most important calculated properties of the macrocycle collection that were potentially synthesized during library assembly. Coloring shows the comparison of the calculated properties with the guidelines from Whitty (blue) and Kihlberg (orange) for potential macrocyclic drugs beyond the rule of five chemical space.

The common rule of five (Ro5) from Lipinski^{[8][9]} cannot be applied for this type of molecules since the occupied chemical space lies beyond the Ro5 which would predict that these compounds are pharmacologically uninteresting. But still, macrocycles show properties that allow them to be outside the Ro5 chemical space and still act as very potent orally bioavailable drugs. Comparing our results with the stated guidelines from **Table 5** we directly see that with the Whitty and the Kihlberg rules fit well with our macrocycle library. The biggest deviation we found with the number of hydrogen bond acceptors (HBAs) in comparison to the Whitty parameters. The majority of our macrocycles possess 9 - 12 HBAs, which is a too low number for the Whitty definition. In contrast the Kihlberg parameters that arose from direct macrocycle measurements states that 15 or less HBAs is fine for a macrocyclic drug. A similar but less drastic picture was found with the topological surface area (TPSA). For both guidelines some fraction of our library lay outside the parameters. Even though in both cases about 75% of our structures fulfilled the criteria. We performed a ring size distribution analysis to further highlight the diversity and potential applicability of the DEML in drug discovery (top left graph in **Figure 17**). The majority of the ring scaffolds contained 16 to 23 ring atoms, even though there were members with very large rings, containing up to 33 ring atoms.

5. DEML Protein Selections and Sequencing Results

5.1 Protein Biotinylation of HSA and AGP

Protein biotinylation was performed according to a modified procedure from Scheuermann et al.^[10] The given procedure below was applied for HSA and AGP modifications.

In a 1.5 ml Eppendorf tube the protein (1 mg/ml in PBS buffer, 1 ml) was mixed with NHS-LC-Biotin (10 mM in DMSO, 20 eq.) and the solution was incubated at 4°C for 3 h (Thermo Block, 500 rpm shaking). The reaction was quenched by the addition of TRIS*HCl buffer (1M, pH 7.42, 66 eq.) and incubated at 4°C for another 1 h. PBS buffer (1 ml) was added and the reaction buffer was exchanged using a Sartorius Vivaspin 2 10000 MWCO CTA column (4200 rpm, 4°C, 4 x 20 min, 3 x 2 ml PBS). The protein solution was collected and PBS was added to get a final protein concentration of 10 µM. The protein solution was aliquoted (20 µL aliquots) and stored in the freezer at -20°C. To check the success of the modification, a biotinylation band shift assay was performed.

The modified protein (7.6 µL) was diluted with PBS (37.4 µL) and mixed with an equimolar amount of avidin (1 mg/ml in PBS, 5 µL). The solution was left standing at RT for 5 min and then placed on ice for 1 h. The result of the experiment was controlled by SDS-PAGE (Coomassie stain). Only indicated control samples were treated by heat denaturation prior to loading.

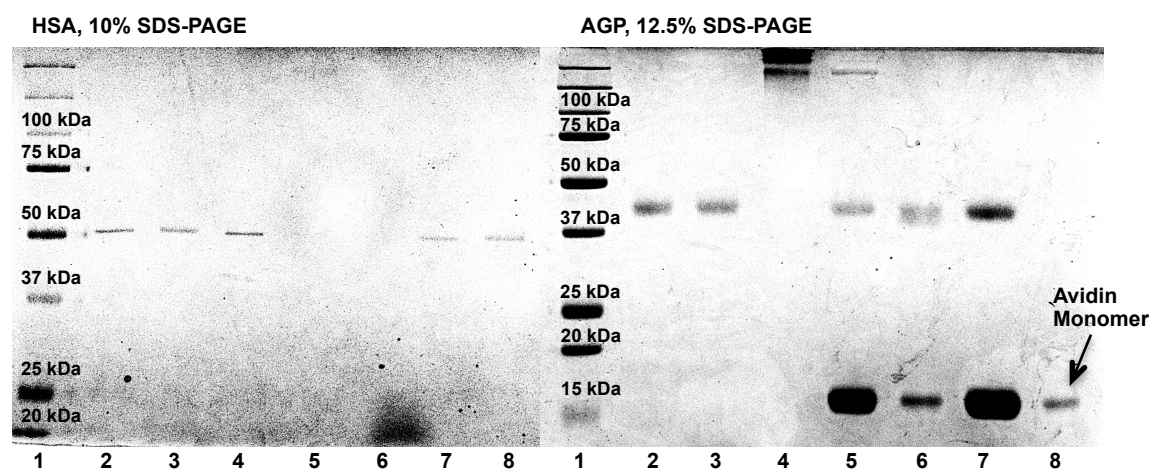


Figure 18. Protein biotinylation band shift assays, Coomassie stain. **Left:** HSA band shift, **L1:** Protein standards, **L2:** HSA unmodified, **L3:** HSA unmodified + avidin, **L4:** HSA modified, **L5:** HSA modified + avidin, **L6:** Avidin, **L7:** HSA unmodified + avidin (95°C), **L8:** HSA modified + avidin (95°C). **Right:** AGP band shift, **L1:** Protein standards, **L2:** AGP unmodified, **L3:** AGP modified, **L4:** AGP modified + avidin, **L5:** AGP modified + avidin (95°C), **L6:** AGP unmodified + avidin, **L7:** AGP unmodified + avidin (95°C), **L8:** Avidin. Avidin tetramer is not visible since it sticks to the stacking gel.

5.2 DEML Protein Target Selections

Stock solutions:

- **Biotin solution:** 200 mM *D*-biotin in DMSO (2 ml)
- **PBST:** 0.05% Tween-20 in PBS (10 ml)
- **PBST-Biotin:** 1:1 mixture of Biotin stock solution + 50% DMSO in PBST (2 ml)-> do not put on ice!
- **Salmon Sperm DNA:** 2 mg/ml in PBS
- **PBST-SS:** PBST containing 0.2 mg/ml Salmon Sperm DNA. Mix 1.8 ml PBST with 0.2 ml Salmon Sperm DNA stock (2 ml).

- **DEML-WS:** DEML working solution. DEML stock (9.4 μ M, 10 μ L) was mixed with PBST-SS (50 μ L) and diluted with PBST (940 μ L) to yield the working solution (1 ml, 94 nM for 10 assays).
- **Protein solution:** biotinylated protein sample (10 μ L, 10 μ M) was diluted with PBS (90 μ L) to the final concentration (1 μ M, 100 μ L)

All stock solutions were prepared as stated above. The DEML-WS and the protein solution were kept on ice. 0.1 mg hydrophilic streptavidin magnetic beads (25 μ L, NEB S1421S) were placed in a magnetic rack and the buffer was discarded. The beads were washed with PBS (3 x 1 ml). The beads were kept in 1 ml PBS on ice until usage.

The buffer was removed from the beads and the protein solution (100 μ L, 100 pmol) was added. The mixture was incubated at 4°C for 30 min while gently mixing by rotation. The tube was placed in the magnetic rack and the buffer was discarded. The beads were suspended in PBST-Biotin (2 x 200 μ L) and the supernatants were removed. The beads were washed with PBST (200 μ L), 10% PBST in DMSO (200 μ L, to remove biotin precipitate) and PBST (2 x 200 μ L). The beads were transferred to a fresh tube and DEML-WS (100 μ L, 9.4 pmol DEML) was added. The mixture was incubated at 4°C for 1 h while gently mixing by rotation. The tube was transferred to the magnetic rack and the supernatant was discarded. The beads were washed with PBST (5 x 200 μ L), exchanging the tube after 2 washing steps (reduce carryover of unbound DNA). The washed beads were suspended in TRIS buffer (10 mM, pH 8.5, 100 μ L) and the bound DNA was eluted from the protein by heat denaturation (95°C for 10 min). After cooling, the magnetic beads were removed and the buffer solution was stored in a 1.5 ml Eppendorf tube at -20°C. PCR amplification and high-throughput sequencing were used for analyzing the target binding results.

Note: For the small DEML the procedure was analog to the described procedure. Due to the higher purity and smaller number of individual members the small DEML-WS was prepared from the 1 μ M small DEML solution, giving a final work solution concentration of 10 nM. Target binding assays were performed in duplicates with two freshly prepared working solutions that were used on two consecutive days. Performed assays with AGP and HSA: 2 x small DEML, 2 x DEML, 4 x dummy selections (beads only, no bound target proteins). In total: 12 selection assays.

5.3 PCR Amplification of Eluted DNA

PCR 1

Eluted DNA from selection:	5 μ L	UniPrimAdapt (7.5 μM):	4 μ L
Phusion HF buffer (5x):	10 μ L	IndexPrimAdapt (10 μM):	3 μ L
Phusion MgCl₂ (50 mM):	2 μ L	Phusion (2U/μL):	0.25 μ L
dNTPs (10 mM):	1.25 μ L	H₂O:	24.5 μ L

In a PCR vial all stock solutions were mixed and kept on ice. The polymerase was added shortly before the PCR program started. 22 PCR cycles were performed. PCR cycle program: 98°C for 2:15 then 22 x 98°C (45 s) → 69°C (45 s) → 72°C (45 s), 72°C (5 min). The progress of the reactions was controlled by Native PAGE analysis (10%, TBE, 150 V, 45 min, SYBR Gold staining). The samples were purified by PCR clean up kit (Macherey&Nagel). Two washing steps were included and the purified PCR products were eluted with NE buffer (2 x 20 μ L). The PCR products were diluted with NE buffer to a final concentration of 100 nM.

For the fingerprint sample, an aliquot of the DEML (small DEML) working solution was diluted 1:10 and processed as stated above.

UniPrimAdapt:	5'-ACACGACGCTCTTCCGATCTGTAGTTGGATCCGCAC-3' (small DEML)
	5'-ACACGACGCTCTTCCGATCTAGAGTATCCATCCGTA-3' (DEML)
IndexPrimAdapt:	5'-GACGTGTGCTCTTCCGATCTGGAGCTTGTGAATTCT-3' (both libraries the same)

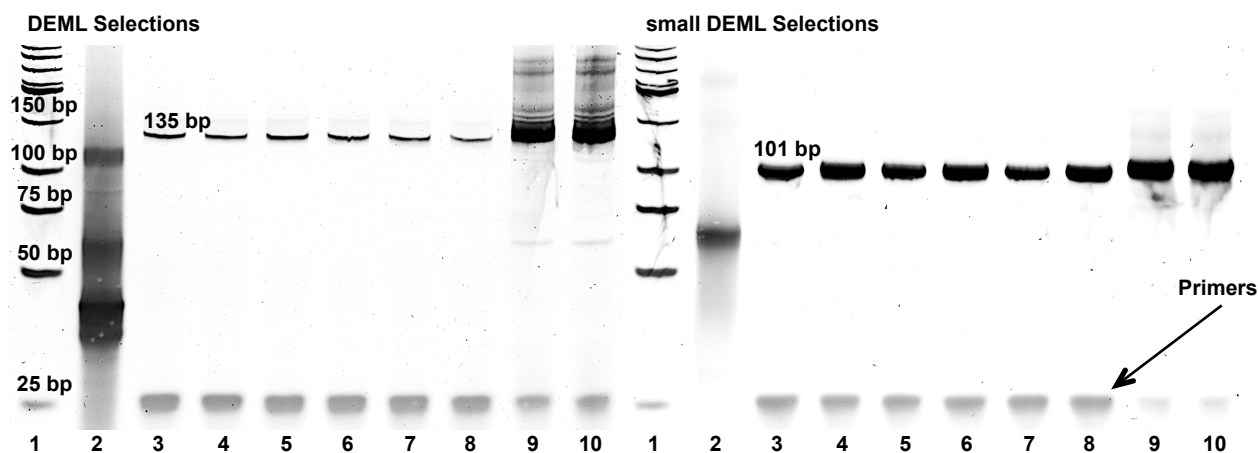


Figure 19. 10% Native PAGE gels after PCR1 with the DEML selections (left) and the small DEML selections (right), SYBR gold staining. L1: Low MW DNA ladder, L2: (small) DEML, L3&L4: HSA selections, L5&L6: Dummy selections, L7&8: AGP selections, L9&10: Library fingerprints.

PCR2

PCR products from PCR1 were diluted 1:10 with NE buffer prior to PCR2

PCR 1 DNA (10 nM):	10 µL	Universal Primer (10 µM):	3 µL
Phusion HF buffer (5x):	20 µL	Index Primer (10 µM):	3 µL
Phusion MgCl ₂ (50 mM):	4 µL	Phusion (2U/µL):	0.5 µL
dNTPs (10 mM):	2.5 µL	H ₂ O:	57 µL

In a PCR vial all stock solutions were mixed and kept on ice. The polymerase was added shortly before the PCR program started. 15 PCR cycles were performed. PCR cycle program: 98°C for 2:15 then 15 x 98°C (45 s) → 69°C (45 s) → 72°C (45 s), 72°C (5 min). The progress of the reactions was controlled by Native PAGE analysis (8%, TBE, 150 V, 45 min, SYBR Gold staining). The samples were purified by Agarose Gel Electrophoresis (2%, TBE, 100 V, 45 min) and the desired bands were cut out of the gel with subsequent DNA isolation with the PCR clean up kit (Macherey&Nagel). Two washing steps were included and the purified PCR products were eluted with NE buffer (2 x 20 µl). After ethanol precipitation the amplified and indexed products were diluted with NE buffer to a final concentration of 100 nM.

Primer sequences for Illumina Sequencing (NEBNext Multiplex Oligos for Illumina, Sets 1 and 3):

UniPrimer:	5'-AATGATACGGCGACCAACGAGATCTACACTCTTTCCTACACGACGCTCTTCCGATCT-3'
IndexPrimers:	5'-CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'

NNNNNN represents the indexing codons (selection ID) defined by the NEB Kits.

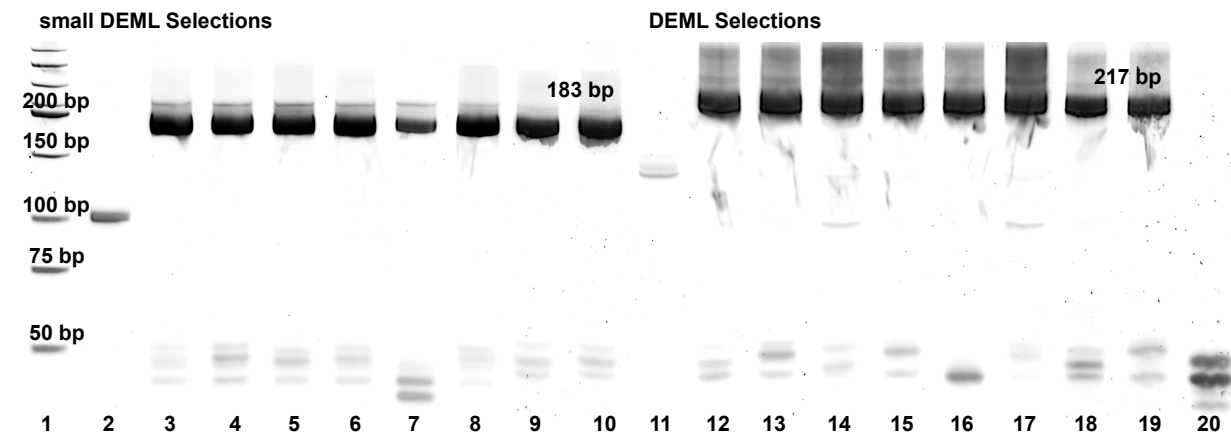


Figure 20. 8% Native PAGE gels after PCR2 amplification. L1: Low MW DNA ladder, L2&L11: PCR1 (HSA selection), L3, L6, L12, L15: HSA selections, L4, L7, L13, L16: Dummy selections, L5, L8, L14, L17: Fingerprints, L9, L10, L18, L19: AGP selections, L20: Primers.

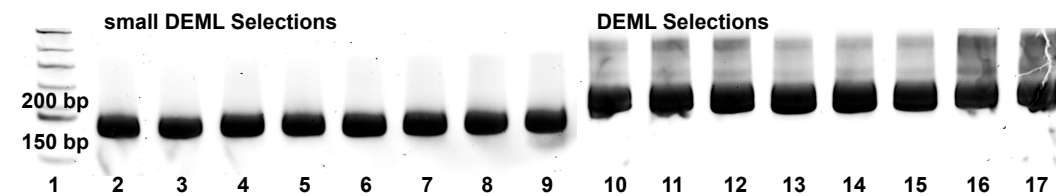


Figure 21. 8% Native PAGE gels after PCR2 purification. L1: Low MW DNA ladder, L2, L3, L10, L11: HSA selections, L4, L5, L12, L13: Dummy selections, L6, L7, L14, L15: AGP selections, L8, L9, L16, L17: Fingerprints.

5.4 Next Generation Sequencing (NGS)

We extracted the codon hit counts from next generation sequencing data files (.fq) with our DECL-Gen software suite on the University of Basel Computational Cluster (SciCore). The script aligns the theoretical DNA strand to the read with the pairwise2 algorithm provided by the Biopython package, and then extracts the codons from the aligned read. This is done for the mate pair, too, and the two codon sets are controlled for identity, discarding the read if they do not match. After extraction, the counts for both replicates have been averaged and the list was cleaned from non-existing codon combinations deriving from random mutations during PCR or from sequencing errors. The results for each data set are summarized in Table 6.

Table 6. Summary of the enrichment assays that were analyzed by NGS. The counts of the different enrichments were averaged by replicate and then normalized by their mean.

Entry	Target	Replicate	Library Size	Reads ^[a]	Matching Codon Pairs ^[b]	Coverage ^[c]	Invalid Codon Content ^[d]	Found Codons ^[e]
1	Fingerprint	1	2'142	15.77	13.41	6260		2142 (100%)
2		2	2'142	20.03	17.38	8114	2.6%	
3	Beads	1	2'142	17.23	15.17	7082	2.1%	2142 (100%)
4		2	2'142	15.14	13.20	6160		
5	HSA	1	2'142	15.81	13.83	6457	2.1%	2142 (100%)
6		2	2'142	15.21	13.28	6200		
7	AGP	1	2'142	17.43	15.22	7106	2.1%	2142 (100%)
8		2	2'142	16.76	14.47	6755		
9	Fingerprint	1	1'420'146	13.07	8.55	6.02	6.7%	1'318'952 (92.9%)
10		2	1'420'146	11.95	7.87	5.54		
11	Beads	1	1'420'146	12.02	8.87	6.25	4.2%	819'242 (57.7%)
12		2	1'420'146	12.30	8.97	6.32		
13	HSA	1	1'420'146	12.33	9.06	6.38	4.3%	838'791 (59.1%)
14		2	1'420'146	13.28	9.77	6.88		
15	AGP	1	1'420'146	10.82	8.02	5.65	4.0%	788'545 (55.5%)
16		2	1'420'146	11.54	8.54	6.01		

[a] Read counts are given in million mate reads. [b] In million reads. Only reads where both read mates have the same codons were considered. [c] Coverage is an indication of how many reads per codon that can be expected if all compounds are distributed equally in the sample. It was calculated by the following equation: "Coverage" = "Matching codon pairs" / "Library Size". [d] This number indicates how many codons were found that did not encode for a compound within a library. [e] Depending on sequencing depth, a part of the library population never gets sequenced in both replicates even if they are equally distributed. A lower number of found codons indicated a strong deviation in the population distribution due to enrichment.

5.5 Results Analysis

Hit identification (smDEML)

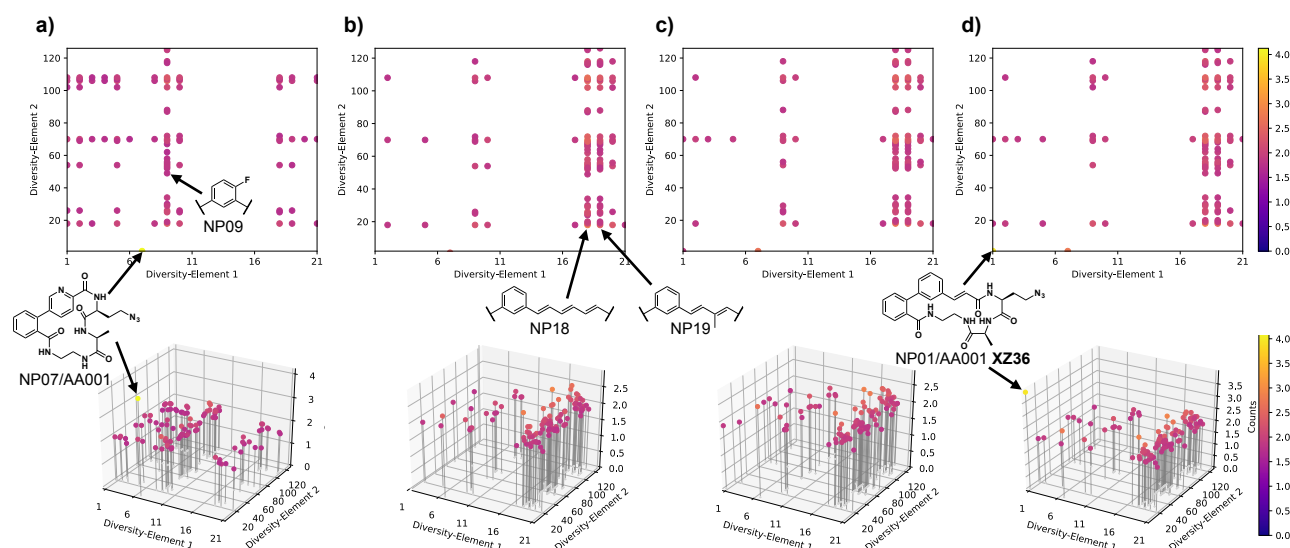


Figure 22. Scatterplots for the selections of the smDEML, presented in 2D (upper) and 3D (lower) plots. For clarity, only the 100 most abundant compounds are shown. **a)** smDEML Fingerprint. **b)** smDEML Dummy selection (beads only). **c)** smDEML selection against AGP. **d)** smDEML selection against HSA.

Potential binders were identified by comparison of the found hits in the AGP and HSA selections to the dummy selection and the fingerprint. Hits containing the DE-1 elements **NP09**, **NP18**, **NP19** and **NP20** were directly excluded, since these compounds were highly overrepresented in the fingerprint and dummy selection. These moieties seemed to favor binding to the magnetic beads (perhaps streptavidin), which led to the conclusion that these compounds were false positives. Macrocycle **NP07/AA001** was found to be the most abundant compound in the fingerprint and reappeared in high amounts in all other selections, which led to its exclusion from the analysis. We created a list of the remaining potential macrocyclic protein binders (see **Table 7**) from which we chose the compounds to be synthesized and tested for their binding affinity. Only the top hit in the HSA selection (MC **NP01/AA001 XZ36**) was finally considered valuable for binding affinity tests. The AGP selection did not reveal any potentially interesting protein binders.

Hit identification (DEML)

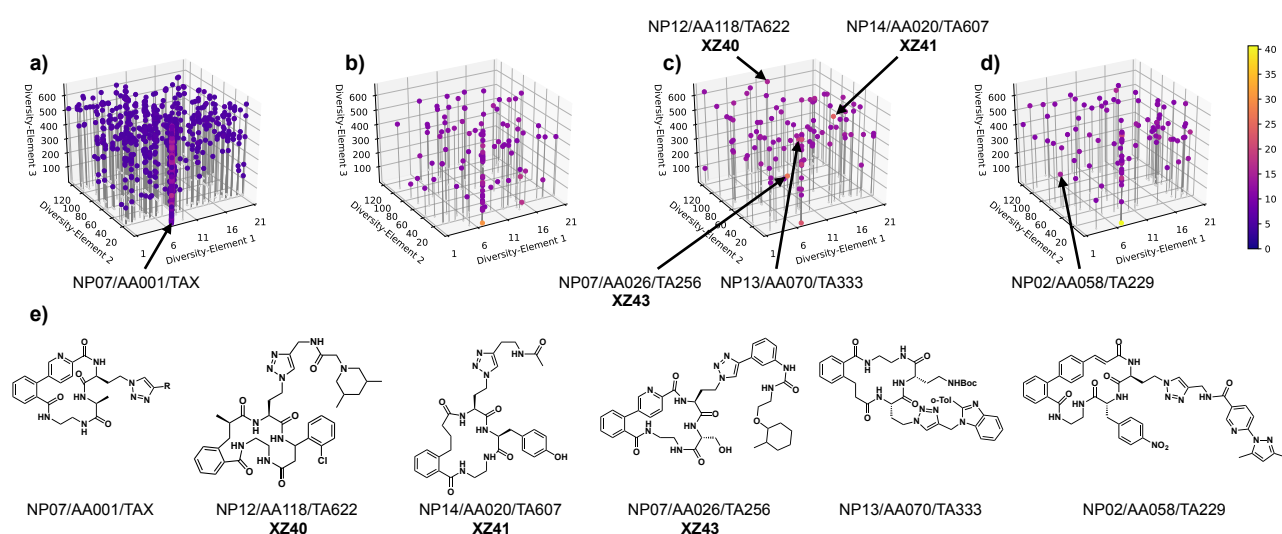


Figure 23. Scatterplots for the selections of the DEML in 3D. For clarity, only the 100 most abundant compounds are shown for the selections, 1000 compounds for the fingerprint plot. **a)** DEML Fingerprint. **b)** DEML Dummy selection (beads only). **c)** DEML selection against AGP. **d)** DEML selection against HSA. **e)** Structures of the indicated compounds (arrows).

As with the smDEML, potential binders were identified by comparison of the found hits in the AGP and HSA selections to the dummy selection and the fingerprint. Hits containing the DE-1 elements **NP09**, **NP18**, **NP19** and **NP20** were excluded again, since these compounds were highly overrepresented in the fingerprint and dummy selection. The compound group NP07/AA001/TAX was highly overrepresented in the fingerprint, resulting from the same high abundance of macrocycle NP07/AA001. A summary of the found top hits of these selections is shown in **Table 7**. We chose four compounds from the AGP selection and one macrocycle from the HSA selection (see **Figure 23** and **Table 7** for the details) to be synthesized and tested for their protein binding affinity. In the case of macrocycle NP02/AA058/TA229 and NP13/AA070/TA333 the first diversity element was changed to NP01 and NP12 respectively. This was done due to the high similarity of these elements and to simplify the synthesis of the macrocycles (see the structures of **XZ42** and **XZ39**).

Table 7. Results for the different library-protein selections. The compounds were ranked after comparison of the protein selection with the dummy selection experiment and the fingerprint. Only the top 6 compounds are listed, the rest was considered too little enriched. Compounds in **bold face** were chosen for resynthesis and binding affinity measurements.

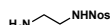
Small DEML selection against HSA					Small DEML selection against AGP				
Rank	DE-1	DE-2	DE-3	Number	Rank	DE-1	DE-2	DE-3	Number
1	NP01	AA001	none	NP1/AA001	1	NP20	AA069	none	NP20/AA069
2	NP18	AA034	none	NP18/AA034	2	NP19	AA088	none	NP19/AA088
3	NP17	AA070	none	NP17/AA070	3	NP19	AA117	none	NP19/AA117
4	NP20	AA069	none	NP20/AA069	4	NP18	AA053	none	NP18/AA053
5	NP19	AA029	none	NP19/AA029					
6	NP20	AA072	none	NP20/AA072					
DEML selection against HSA					DEML selection against AGP				
Rank	DE-1	DE-2	DE-3	Number	Rank	DE-1	DE-2	DE-3	Number
1	NP21	AA026	TA423	NP21/AA026/TA423	1	NP07	AA026	TA256	NP07/AA026/TA256
2	NP14	AA086	TA621	NP14/AA086/TA621	2	NP14	AA020	TA607	NP14/AA020/TA607
3	NP02^[a]	AA058	TA229	NP02/AA058/TA229	3	NP13^[b]	AA070	TA333	NP13/AA070/TA333
4	NP15	AA020	TA459	NP15/AA020/TA459	4	NP12	AA118	TA622	NP12/AA118/TA622

5	NP19	AA039	TA357	NP19/AA039/TA357	5	NP21	AA125	TA412	NP21/AA125/TA412
6	NP12	AA029	TA553	NP12/AA029/TA553	6	NP09	AA029	TA443	NP09/AA029/TA443

[a] Due to the high similarity and ease of chemical synthesis, NP02 was replaced by NP01 for off-DNA macrocycle synthesis. [b] Due to the high similarity and ease of chemical synthesis, NP13 was replaced by NP12 for off-DNA macrocycle synthesis.

6. Macrocycle Resynthesis

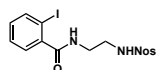
6.1 *N*-(2-aminoethyl)-2-nitrobenzenesulfonamide **XZ1**



A solution of 2-nitrobenzenesulfonyl chloride (25.0 g, 113.0 mmol, 1.0 eq.) in DCM (250 ml) was added dropwise to a cooled solution of ethylenediamine (75.4 mL, 1.1 mol, 10.0 eq.) in DCM (250 ml) over 1.5 h. The mixture was stirred at RT for 3 h. The yellow mixture was washed with H₂O (3 x 200 ml), brine (150 ml) and the organic layer was dried over Na₂SO₄ and concentrated (residual ethylenediamine was removed by hv) to yield the desired product as a thick yellow oil (12.2 g, **44%**). The material was used without further purification for the next step. Analytical data was in agreement with reported data.^[11]

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 8.17 – 8.12 (m, 1H), 7.88 – 7.84 (m, 1H), 7.75 (m, 2H), 3.12 (dd, *J* = 6.5, 5.0 Hz, 2H), 2.87 (dd, *J* = 6.5, 4.9 Hz, 2H).

6.2 2-Iodo-*N*-(2-((2-nitrophenyl)sulfonamido)ethyl)benzamide **XZ2** (Nos-Sc-I)



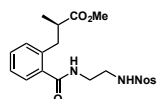
In a 250 mL flask 2-iodobenzoic acid (8.2 g, 33.1 mmol, 1.0 eq.), **XZ1** (12.2 g, 49.6 mmol, 1.5 eq.), HBTU (15.0 g, 39.7 mmol, 1.2 eq.) and DIPEA (17.3 mL, 99.2 mmol, 3.0 eq.) were dissolved in THF (150 mL) and stirred at RT for 2 h after which UPLC-MS analysis showed 93% conversion to the desired product (HBTU dissolved after about 30 min reaction time). The solvent was removed *in vacuo* and the residue was dissolved in EtOAc (250 mL). The organic layer was washed with diluted HCl (20 mM, 3 x 100 mL), half-saturated NaHCO₃ (3 x 100 mL) and brine (100 mL). The organic layer was dried over MgSO₄ and concentrated. The crude was purified by flash column chromatography on the ISOLERA (Silica, 2 x 340 g, cyclohexane:EtOAc 20% -> 70% EtOAc, UV). The product containing fractions were combined and concentrated. The obtained solid was dissolved in MeCN (100 mL), filtered over a G4 glass sintered funnel and concentrated to yield the desired product as a yellow solid (14.7 g, **94%**).

¹H NMR (400 MHz, Acetonitrile-*d*₃) δ/ppm: 8.10 – 8.03 (m, 1H), 7.91 – 7.83 (m, 2H), 7.83 – 7.76 (m, 2H), 7.41 (ddd, *J* = 7.5, 7.5, 1.1 Hz, 1H), 7.32 (dd, *J* = 7.6, 1.7 Hz, 1H), 7.14 (ddd, *J* = 7.9, 7.4, 1.8 Hz, 1H), 6.90 (brs, 1H), 6.21 (t, *J* = 5.9 Hz, 1H), 3.44 (dt, *J* = 5.8, 6.0 Hz, 2H), 3.30 – 3.22 (dt, 6.0, 6.1 Hz, 2H).

¹³C NMR (101 MHz, CD₃CN) δ/ppm: 170.75, 143.29, 140.57, 135.19, 135.10, 133.88, 133.80, 131.97, 131.40, 129.15, 128.88, 126.02, 93.03, 43.98, 40.27.

HRMS (ESI): C₁₅H₁₄IN₃NaO₅S⁺ *calcd*: 497.9591, *found*: 497.9597.

6.3 Nos-Sc-NP12-OMe **XZ3**



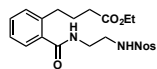
Under an inert atmosphere **XZ2** (3.0 g, 6.3 mmol, 1.0 eq.) and Pd (π-cinnamyl) chloride dimer (327.0 mg, 631.0 μmol, 10 mol%) were dissolved in THF (60 mL) and the solution was cooled to 0°C. A solution of (S)-3-Methoxy-2-methyl-3-oxopropylzinc bromide (0.5 M in THF, 18.9 mL, 9.5 mmol, 1.5 eq.) was slowly added and the solution was stirred at 0°C for 15 min. The cooling bath was removed and the mixture was stirred at RT for 45 min after which UPLC analysis showed full consumption of the starting material. The reaction was stopped by the addition of H₂O (5 mL) and the mixture was filtered over G4. The volatiles were removed *in vacuo* and the residue was dissolved in EtOAc (150 mL) and washed with half-saturated NH₄Cl (100 mL) and H₂O (2 x 100 mL). The combined aqueous layers were extracted with EtOAc (2 x 100 mL) and the combined organic layers were treated with brine (100 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 340g, cyclohexane:EtOAc, UV). The product containing fractions were combined and concentrated to yield the desired product as a thick, slightly red oil (2.3 g, **80%**).

^1H NMR (400 MHz, Acetonitrile- d_3) δ /ppm: 8.11 – 8.02 (m, 1H), 7.88 – 7.83 (m, 1H), 7.83 – 7.76 (m, 2H), 7.38 – 7.31 (m, 2H), 7.29 – 7.18 (m, 2H), 6.96 (s, 1H), 6.30 (t, J = 5.7 Hz, 1H), 3.53 (s, 3H), 3.48 – 3.38 (m, 2H), 3.25 (dt, J = 5.7, 5.9 Hz, 2H), 3.12 – 3.03 (m, 1H), 2.85 – 2.74 (m, 2H), 1.07 (d, J = 6.6 Hz, 3H).

^{13}C NMR (101 MHz, CD_3CN) δ /ppm: 177.33, 171.17, 148.94, 138.72, 137.49, 135.06, 133.82, 133.81, 131.55, 131.40, 130.65, 128.20, 127.28, 125.96, 52.04, 44.12, 41.99, 40.24, 37.43, 17.53.

HRMS (ESI): $\text{C}_{20}\text{H}_{23}\text{N}_3\text{NaO}_7\text{S}^+$ *calcd*: 472.1149, *found*: 472.1152.

6.4 Nos-Sc-NP14-OEt XZ4



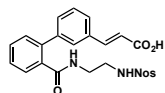
Under an inert atmosphere **XZ2** (3.0 g, 6.3 mmol, 1.0 eq.) and Pd (π -cinnamyl) chloride dimer (327.0 mg, 631.0 μmol , 10 mol%) were dissolved in THF (60 mL) and the solution was cooled to 0°C. 4-Ethoxy-4-oxobutylzinc bromide solution (0.5 M in THF, 18.9 mL, 9.5 mmol, 1.5 eq.) was slowly added and the solution was stirred at 0°C for 15 min. The cooling bath was removed and the mixture was stirred at RT for 45 min after which UPLC analysis showed full consumption of the starting material. The reaction was stopped by the addition of H_2O (5 mL). The mixture was filtered and the volatiles were removed *in vacuo*. The residue was dissolved in EtOAc (150 mL) and washed with half-saturated NH_4Cl (100 mL) and H_2O (2 x 100 mL). The combined aqueous layers were extracted with EtOAc (2 x 100 mL) and the combined organic layers were treated with brine (100 mL), dried over Na_2SO_4 and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 340g, cyclohexane:EtOAc, UV) to yield the desired product as a thick, slightly yellow oil (2.4 g, 82%).

^1H NMR (400 MHz, Acetonitrile- d_3) δ /ppm 8.09 – 8.03 (m, 1H), 7.88 – 7.83 (m, 1H), 7.82 – 7.76 (m, 2H), 7.35 (td, J = 7.5, 1.5 Hz, 1H), 7.30 (dd, J = 7.6, 1.4 Hz, 1H), 7.25 (ddd, J = 7.6, 1.3, 0.6 Hz, 1H), 7.21 (td, J = 7.4, 1.4 Hz, 1H), 6.91 (s, 1H), 6.31 (t, J = 5.7 Hz, 1H), 4.04 (q, J = 7.1 Hz, 2H), 3.44 (q, J = 5.9 Hz, 2H), 3.27 – 3.21 (m, 2H), 2.76 – 2.68 (m, 2H), 2.24 (t, J = 7.4 Hz, 2H), 1.88 – 1.78 (m, 2H), 1.19 (t, J = 7.1 Hz, 3H).

^{13}C NMR (101 MHz, CD_3CN) δ /ppm 174.10, 171.32, 140.76, 137.42, 135.06, 133.82, 131.40, 131.01, 130.71, 129.37, 127.99, 127.94, 126.87, 125.95, 60.89, 44.21, 40.16, 34.38, 32.98, 27.52, 14.54.

HRMS (ESI): $\text{C}_{21}\text{H}_{25}\text{N}_3\text{NaO}_7\text{S}^+$ *calcd*: 486.1305, *found*: 486.1309.

6.5 Nos-Sc-NP01-OH XZ5



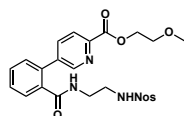
In a 250 mL flask **XZ2** (3.0 g, 6.3 mmol, 1.0 eq.), 3-(2-carboxyvinyl)benzeneboronic acid (1.8 g, 9.5 mmol, 1.5 eq.), Pd (π -cinnamyl) chloride dimer (327.0 mg, 631.0 μmol , 10 mol%) and K_3PO_4 (4.0 g, 18.9 mmol, 3.0 eq.) were dissolved in EtOH (56 mL) and H_2O (24 mL). The solution was stirred at 50°C for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed and the residue was taken up in EtOAc (200 mL) and H_2O (150 mL). The mixture was acidified with concentrated HCl to pH 1. The biphasic mixture was separated and the aqueous layer was extracted with EtOAc (2 x 100 mL, 1 x 50 mL). The combined organic layers were washed with brine (100 mL), dried over Na_2SO_4 and were concentrated by rotary evaporation. The crude was purified by reversed phase flash chromatography on the ISOLERA (RP-Silica, 340 g, $\text{H}_2\text{O}/\text{MeCN}$ + 0.1% TFA, UV). The product-containing fractions were freeze dried to yield the desired product as off-white powder (2.2 g, 70%).

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.22 (t, J = 5.8 Hz, 1H), 8.11 (t, J = 5.9 Hz, 1H), 7.99 (dd, J = 5.9, 3.4 Hz, 1H), 7.96 – 7.92 (m, 1H), 7.91 – 7.86 (m, 2H), 7.64 – 7.54 (m, 3H), 7.54 – 7.48 (m, 1H), 7.47 – 7.40 (m, 3H), 7.38 – 7.30 (m, 2H), 6.50 (d, J = 16.0 Hz, 1H), 3.14 (dt, J = 7.6, 6.0 Hz, 2H), 2.77 (dt, J = 7.6, 6.2 Hz, 2H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 169.19, 167.48, 147.70, 143.68, 140.77, 138.44, 136.94, 134.10, 132.71, 132.54, 130.14, 129.81, 129.51, 129.38, 128.63, 127.94, 127.63, 127.34, 126.99, 124.57, 119.46, 41.56, 38.78.

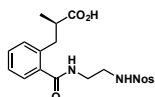
HRMS (ESI): $\text{C}_{24}\text{H}_{21}\text{N}_3\text{NaO}_7\text{S}^+$ *calcd*: 518.0992, *found*: 518.0991.

6.6 Nos-Sc-NP07-OEtOMe XZ6



In a 100 mL flask **XZ2** (1.5 g, 3.2 mmol, 1.0 eq.), 2-(methylcarboxy)pyridine-5-boronic acid pinacol ester (1.0 g, 3.8 mmol, 1.2 eq.), Pd(dppf)Cl₂ (348.0 mg, 475.0 μmol, 15 mol%) and triethylamine (883.0 μL, 6.3 mmol, 2.0 eq.) were dissolved in methoxyethanol (40 mL) and the mixture was stirred at 100°C for 4 h after which UPLC-MS analysis showed full conversion to the desired product as methoxyethylester. The solvent was removed *in vacuo* and the crude was taken up in EtOAc (100 mL). The organic layer was washed with diluted HCl (pH 1, 3 x 100 mL). The combined aqueous layers were extracted with EtOAc (3 x 100 mL) and the combined organic layers were washed with brine (100 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, H₂O/MeCN + 0.1% TFA, UV). The product-containing fractions were lyophilized to yield the desired product as dark brown waxy solid (884 mg, **53%**). NMR analysis showed co-elution of some sideproducts. The product was used without further purification for the next steps. Characterization of the compound was performed after ester saponification to compound **XZ9**.

6.7 Nos-Sc-NP12-OH XZ7



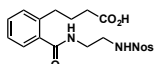
In a 100 mL flask **XZ3** (2.2 g, 4.9 mmol, 1.0 eq.) was dissolved in MeCN (30 mL) and a solution of LiOH · H₂O (1.0 g, 24.7 mmol, 5.0 eq.) in H₂O (15 mL) was added. The mixture was stirred at RT for 2.5 h after which UPLC-MS analysis showed complete conversion to the desired product. Acidifying with concentrated HCl to pH 5-6 quenched the reaction. The volatiles were removed *in vacuo* and the aqueous residue was taken up in EtOAc (150 mL) and H₂O (100 mL). The mixture was acidified to pH 1 with concentrated HCl. The layers were separated (addition of brine for good separation) and the aqueous layer was extracted with EtOAc (2 x 100 mL). The combined organic layers were washed with half-saturated brine (2 x 100 mL) and brine (100 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The desired product was obtained as an off-white solid (2.2 g, **quant. 93% HPLC purity**).

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 12.06 (s, 1H), 8.34 (t, *J* = 5.7 Hz, 1H), 8.18 (t, *J* = 5.9 Hz, 1H), 8.06 – 8.00 (m, 1H), 8.00 – 7.95 (m, 1H), 7.91 – 7.83 (m, 2H), 7.37 – 7.31 (m, 2H), 7.28 – 7.19 (m, 2H), 3.35 – 3.26 (m, 2H), 3.12 – 3.05 (m, 2H), 3.02 (dd, *J* = 13.3, 6.9 Hz, 1H), 2.72 (dd, *J* = 13.3, 7.6 Hz, 1H), 2.67 – 2.57 (m, 1H), 0.97 (d, *J* = 6.9 Hz, 3H).

¹³C NMR (101 MHz, DMSO) δ/ppm 177.00, 169.29, 147.70, 137.53, 136.89, 134.08, 132.72, 132.67, 130.29, 129.48, 129.25, 127.38, 125.99, 124.51, 42.04, 40.28, 39.00, 36.05, 16.90.

HRMS (ESI): C₁₉H₂₂N₃O₇S⁺ *calcd*: 436.1173, *found*: 436.1167.

6.8 Nos-Sc-NP14-OH XZ8



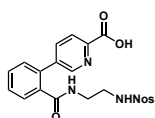
In a 100 mL flask **XZ4** (2.3 g, 4.9 mmol, 1.0 eq.) was dissolved in MeCN (30 mL) and a solution of LiOH · H₂O (1.0 g, 24.6 mmol, 5.0 eq.) in H₂O (15 mL) was added. The mixture was stirred at RT for 2.5 h after which UPLC-MS analysis showed complete conversion to the desired product. Acidifying with concentrated HCl to pH 5-6 quenched the reaction. The volatiles were removed and the aqueous residue was taken up in EtOAc (150 mL) and H₂O (100 mL). The mixture was acidified to pH 1 with concentrated HCl. The layers were separated (addition of brine for good separation) and the aqueous layer was extracted with EtOAc (2 x 100 mL). The combined organic layers were washed with half-saturated brine (2 x 100 mL) and brine (100 mL), dried over Na₂SO₄ and concentrated by rotary evaporation to yield the desired product as yellow, waxy solid (2.1 g, **quant. 73% HPLC purity**).

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 12.00 (s, 1H), 8.30 (t, *J* = 5.7 Hz, 1H), 8.17 (t, *J* = 5.9 Hz, 1H), 8.05 – 8.01 (m, 1H), 8.00 – 7.97 (m, 1H), 7.89 – 7.85 (m, 2H), 7.38 – 7.29 (m, 2H), 7.28 – 7.17 (m, 2H), 3.34 – 3.28 (m, 2H), 3.11 – 3.05 (m, 2H), 2.74 – 2.58 (m, 2H), 2.17 (t, *J* = 7.5 Hz, 2H), 1.82 – 1.65 (m, 2H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 174.24, 169.35, 147.71, 139.37, 136.80, 134.07, 132.72, 132.68, 129.48, 129.35, 128.22, 127.22, 125.66, 124.49, 42.09, 38.97, 33.35, 31.85, 26.32.

HRMS (ESI): C₁₉H₂₂N₃O₇S⁺ *calcd*: 436.1173, *found*: 436.1166.

6.9 Nos-Sc-NP07-OH XZ9



In a 100 mL flask **XZ6** (842 mg, 1.6 mmol, 1.0 eq.) was dissolved in MeCN (10 mL) and a solution of LiOH·H₂O (334 mg, 8.0 mmol, 5.0 eq.) in H₂O (5 mL) was added. The mixture was stirred at RT for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. Acidifying with concentrated HCl to pH 2 quenched the reaction. The volatiles were removed *in vacuo* and the aqueous residue was taken up in EtOAc (50 mL) and H₂O (50 mL). The mixture was acidified to pH 1 with concentrated HCl. The layers were separated (addition of brine for good separation) and the aqueous layer was extracted with EtOAc (2 x 50 mL). The combined organic layers were washed with half-saturated brine (2 x 50 mL) and brine (50 mL), dried over Na₂SO₄ and were concentrated by rotary evaporation. The crude was purified by reversed phase flash column chromatography (RP-Silica, 340 g, 0.1% TFA in H₂O/MeCN). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (366 mg, 49%).

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 8.65 (dd, *J* = 2.3, 0.8 Hz, 1H), 8.46 (t, *J* = 5.7 Hz, 1H), 8.15 (t, *J* = 6.0 Hz, 1H), 8.03 (dd, *J* = 8.1, 0.8 Hz, 1H), 8.02 – 7.94 (m, 2H), 7.92 (dd, *J* = 8.0, 2.3 Hz, 1H), 7.90 – 7.83 (m, 2H), 7.65 – 7.45 (m, 4H), 3.23 – 3.13 (m, 2H), 2.96 – 2.85 (m, 2H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 168.59, 165.94, 148.46, 147.68, 146.72, 139.05, 136.93, 136.76, 135.18, 134.13, 132.76, 132.58, 130.18, 130.06, 129.46, 128.41, 128.12, 124.55, 124.18, 41.77, 39.02.

HRMS (ESI): C₂₁H₁₉N₄O₇S⁺ *calcd*: 471.0969, *found*: 471.0966.

6.10 Imidazole-1-sulfonyl azide hydrochloride **XZ10**

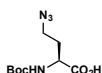


Synthesis according to a literature reported procedure.^[12]

Under an inert atmosphere NaN₃ (3.9 g, 60.0 mmol, 1.0 eq.) was suspended in MeCN (60 mL) and cooled to 0°C in an ice bath. Sulfuryl chloride (4.9 mL, 60.0 mmol, 1.0 eq.) was added dropwise over 10 min. The mixture was stirred at RT for 18 h. After that, the mixture was cooled in an ice-bath and imidazole (7.8 g, 114.0 mmol, 1.9 eq.) was added portionwise. The mixture was continued stirring at RT for 3 h. EtOAc (120 mL) was added and the mixture was washed with H₂O (2 x 120 mL), saturated NaHCO₃ (2 x 120 mL), dried over Na₂SO₄ and filtered. A solution of HCl in EtOH (prepared by the addition of AcCl (6.42 mL, 90 mmol) to dry EtOH (22.5 mL) while cooling) was added dropwise to the filtrate. The mixture was cooled in an ice bath and stirred for 1 h. The formed solid was filtered off, washed with EtOAc (3 x 10 mL) and dried at hv to yield the desired product as white solid (9.1 g, 72%). Analytical data was in agreement with reported data.

¹H NMR (400 MHz, Deuterium Oxide) δ/ppm: 9.44 (t, *J* = 1.4 Hz, 1H), 8.06 (dd, *J* = 2.2, 1.6 Hz, 1H), 7.65 (dd, *J* = 2.2, 1.2 Hz, 1H).

6.11 Boc-Dab(N₃)-OH **XZ11**



The compound was synthesized according to a modified procedure by E.D. Goddard-Borger and R.V. Stick.^[12] Compound is also commercially available as CHA salt.

Under an inert atmosphere Boc-Dab-OH (5.9 g, 27.2 mmol, 1.0 eq.) and **XZ10** (6.8 g, 32.7 mmol, 1.2 eq) were dissolved in MeOH (140 mL) and potassium carbonate (10.2 g, 73.5 mmol, 2.7 eq.) was added, followed by CuSO₄ pentahydrate (68.0 mg, 272 μmol, 1 mol%). The blue mixture was stirred at RT for 2 h after which TLC (5% MeOH in DCM, Ninhydrin) showed complete consumption of the starting material. The solvent was removed and the crude was dissolved in EtOAc (150 mL) and H₂O (150 mL). The biphasic mixture was acidified with concentrated HCl to pH 1-2. The biphasic mixture separated and the aqueous layer was extracted with EtOAc (2 x 150 mL) and the combined organic layers were washed with H₂O (2 x 150 mL), brine (150 mL), dried over Na₂SO₄ and concentrated by rotary evaporation to yield the desired product as slightly yellow oil (7.9 g, >100%). NMR analysis showed some unidentified peaks. The material was used as obtained without further purification.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 12.60 (s, 1H), 3.96 (ddd, *J* = 9.9, 8.2, 4.5 Hz, 1H), 3.42 (ddd, *J* = 12.4, 7.2, 5.4 Hz, 1H), 3.34 (ddd, *J* = 12.3, 8.0, 6.5 Hz, 1H), 1.97 – 1.84 (m, 1H), 1.85 – 1.71 (m, 1H), 1.38 (s, 9H).

6.12 H-Dab(N₃)-OMe **XZ12**



Under an inert atmosphere **XZ11** (7.9 g, 32.5 mmol, 1.0 eq.) was dissolved in MeOH (150 mL) and cooled in an ice bath. Thionyl chloride (19.0 mL, 260.0 mmol, 8.0 eq.) was added dropwise and the solution was stirred at 0°C for 15 min. The cooling bath was removed and the solution was stirred at RT for 24 h after which TLC (BAW, Ninhydrin, R_f=0.58) and NMR showed complete

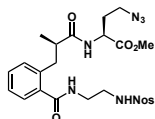
conversion to the desired product. The solvent was removed by rotary evaporation and the residue was dissolved in half-saturated NaHCO₃ solution (200 mL). The aqueous solution was extracted with EtOAc (10 x 100 mL) and the combined organic layers were dried over Na₂SO₄ and concentrated to yield the desired product as yellow liquid which solidified over time in the fridge (3.4 g, **66%**).

¹H NMR (400 MHz, Methanol-*d*₄) δ/ppm: 3.74 (s, 3H), 3.55 (dd, *J* = 7.5, 5.6 Hz, 1H), 3.45 (td, *J* = 6.6, 1.5 Hz, 2H), 1.98 (dddd, *J* = 14.0, 7.3, 6.8, 5.6 Hz, 1H), 1.81 (ddt, *J* = 14.0, 7.5, 6.4 Hz, 1H).

¹³C NMR (101 MHz, MeOD) δ/ppm: 176.65, 52.75, 52.60, 49.85, 34.47.

HRMS (ESI): C₅H₁₁N₄O₂⁺ *calcd*: 159.0877, *found*: 159.0876.

6.13 Nos-Sc-NP12-TFL2(N₃)-OMe XZ13



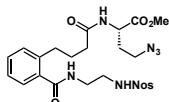
In a 100 mL flask **XZ7** (2.2 g, 4.9 mmol, 1.0 eq.), **XZ12** (937.0 mg, 5.9 mmol, 1.2 eq.), HBTU (2.2 g, 5.9 mmol, 1.2 eq.) and DIPEA (2.6 mL, 14.8 mmol, 3.0 eq.) were dissolved in THF (42 mL) and the mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The solvent was removed by rotary evaporation and the crude mixture was purified by flash column chromatography on the ISOLERA (Silica, 340 g, DCM/MeOH, UV). Product-containing fractions were combined and concentrated to yield the desired product as a thick, yellow oil (4.0 g, **>100%**). NMR showed co-eluted byproducts from the coupling reagent. The material was used without further purification for the next synthetic step.

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 8.13 – 8.05 (m, 1H), 7.81 – 7.74 (m, 1H), 7.75 – 7.66 (m, 2H), 7.58 (t, *J* = 5.8 Hz, 1H), 7.41 – 7.28 (m, 2H), 7.25 – 7.16 (m, 2H), 6.79 (d, *J* = 8.1 Hz, 1H), 6.75 (t, *J* = 6.0 Hz, 1H), 4.53 (td, *J* = 8.3, 4.6 Hz, 1H), 3.67 (s, 3H), 3.66 – 3.61 (m, 1H), 3.54 – 3.44 (m, 1H), 3.42 – 3.30 (m, 2H), 3.19 – 3.05 (m, 2H), 2.87 – 2.80 (m, 2H), 2.71 – 2.63 (m, 1H), 1.88 (dddd, *J* = 14.2, 7.7, 6.6, 4.7 Hz, 1H), 1.66 (ddt, *J* = 14.5, 8.4, 6.2 Hz, 1H), 1.21 (d, *J* = 6.7 Hz, 3H).

¹³C NMR (101 MHz, CDCl₃) δ/ppm: 176.43, 172.10, 171.06, 148.07, 137.35, 136.20, 133.75, 133.67, 132.81, 130.84, 130.32, 130.19, 127.94, 126.69, 125.15, 52.66, 49.60, 47.10, 43.39, 43.22, 43.04, 40.39, 31.04, 18.21.

HRMS (ESI): C₂₄H₂₉N₇NaO₈S⁺ *calcd*: 598.1691, *found*: 598.1693.

6.14 Nos-Sc-NP14-TFL2(N₃)-OMe XZ14



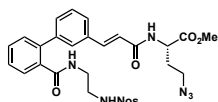
In a 100 mL flask **XZ8** (2.1 g, 4.9 mmol, 1.0 eq.), **XZ12** (925.0 mg, 5.9 mmol, 1.2 eq.), HBTU (2.2 g, 5.9 mmol, 1.2 eq.) and DIPEA (2.5 mL, 14.6 mmol, 3.0 eq.) were dissolved in THF (42 mL) and the mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was dissolved in EtOAc (100 mL) and H₂O (100 mL). The mixture was acidified to pH 1 by addition of concentrated HCl. The layers were separated and the aqueous phase was extracted with EtOAc (2 x 100 mL). The combined organic layers were washed with H₂O (100 mL), brine (100 mL), dried over MgSO₄ and concentrated by rotary evaporation. The crude material was purified by flash column chromatography on the ISOLERA (Silica, 340 g, DCM/MeOH, UV) to yield the desired product as yellow oil (2.7 g, **96%**).

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 8.13 – 8.07 (m, 1H), 7.81 – 7.76 (m, 1H), 7.72 – 7.69 (m, 2H), 7.33 – 7.27 (m, 2H), 7.21 – 7.13 (m, 2H), 6.84 (t, *J* = 5.9 Hz, 1H), 6.74 (d, *J* = 7.8 Hz, 1H), 6.60 (t, *J* = 5.8 Hz, 1H), 4.59 (td, *J* = 7.7, 5.1 Hz, 1H), 3.71 (s, 3H), 3.62 – 3.55 (m, 2H), 3.37 – 3.30 (m, 4H), 2.83 – 2.75 (m, 2H), 2.29 (t, *J* = 7.0 Hz, 2H), 2.08 (dtd, *J* = 14.0, 6.9, 5.1 Hz, 1H), 1.98 – 1.89 (m, 3H).

¹³C NMR (101 MHz, CDCl₃) δ/ppm: 173.52, 172.40, 171.16, 148.11, 139.75, 135.81, 133.46, 132.81, 130.98, 130.38, 130.28, 127.30, 127.17, 126.24, 125.21, 52.67, 50.15, 47.90, 43.44, 39.80, 35.67, 32.65, 31.25, 27.06.

HRMS (ESI): C₂₄H₂₉N₇NaO₈S⁺ *calcd*: 598.1691, *found*: 598.1701.

6.15 Nos-Sc-NP01-TFL2(N₃)-OMe XZ15



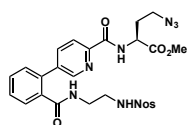
In a 500 mL flask **XZ5** (2.2 g, 4.4 mmol, 1.0 eq.), **XZ12** (841.0 mg, 5.3 mmol, 1.2 eq.), HBTU (2.0 g, 5.3 mmol, 1.2 eq.) and DIPEA (2.3 mL, 13.3 mmol, 3.0 eq.) were dissolved in THF (40 mL) and the mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the crude was purified by flash column chromatography on the ISOLERA (Silica, 340 g, DCM/MeOH, UV) to yield the desired product as a thick, yellow oil (3.7 g, >100%). NMR showed co-eluted byproducts from the coupling reagents. The material was used without further purification for the next synthetic step.

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 8.01 – 7.93 (m, 1H), 7.80 – 7.73 (m, 1H), 7.73 – 7.65 (m, 2H), 7.58 – 7.25 (m, 9H), 7.00 (d, *J* = 7.8 Hz, 1H), 6.54 (d, *J* = 15.7 Hz, 1H), 6.50 (t, *J* = 5.8 Hz, 1H), 5.92 (t, *J* = 5.9 Hz, 1H), 4.70 (td, *J* = 7.9, 5.0 Hz, 1H), 3.70 (s, 3H), 3.37 (t, *J* = 6.7 Hz, 2H), 3.33 – 3.25 (m, 2H), 3.01 – 2.93 (m, 2H), 2.18 – 2.07 (m, 1H), 2.03 – 1.90 (m, 1H).

¹³C NMR (101 MHz, CDCl₃) δ/ppm: 172.26, 170.70, 166.13, 147.93, 140.97, 140.89, 139.09, 135.47, 135.00, 133.92, 133.07, 132.98, 130.90, 130.44, 130.25, 130.11, 129.18, 128.23, 127.87, 127.75, 127.61, 125.30, 121.03, 52.68, 50.34, 47.81, 43.06, 39.85, 31.19.

HRMS (ESI): C₂₉H₂₉N₇NaO₈S⁺ *calcd*: 658.1691, *found*: 658.1693.

6.16 Nos-Sc-NP07-TFL2(N₃)-OMe **XZ16**



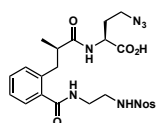
In a 50 mL flask **XZ9** (315.0 mg, 670.0 μmol, 1.0 eq.), **XZ12** (127.0 mg, 803.0 μmol, 1.2 eq.), HBTU (305.0 g, 803.0 μmol, 1.2 eq.) and DIPEA (350.0 μL, 2.0 mmol, 3.0 eq.) were dissolved in THF (5 mL) and DMF (1 mL) and the mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was dissolved in EtOAc (50 mL) and H₂O (50 mL). The mixture was acidified to pH 1 by addition of concentrated HCl. The layers were separated and the aqueous phase was extracted with EtOAc (2 x 50 mL). The combined organic layers were washed with H₂O (50 mL), brine (50 mL), dried over MgSO₄ and concentrated by rotary evaporation. The crude material was purified by flash column chromatography on the ISOLERA (Silica, 120 g, DCM/MeOH, UV) to yield the desired product as a yellowish solid (397 mg, 97%).

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 8.67 (d, *J* = 8.3 Hz, 1H), 8.65 (dd, *J* = 2.3, 0.9 Hz, 1H), 8.19 (dd, *J* = 8.0, 0.8 Hz, 1H), 8.09 – 8.04 (m, 1H), 7.93 (dd, *J* = 8.0, 2.2 Hz, 1H), 7.87 – 7.79 (m, 1H), 7.80 – 7.68 (m, 2H), 7.64 (dd, *J* = 7.6, 1.2 Hz, 1H), 7.54 (td, *J* = 7.6, 1.5 Hz, 1H), 7.47 (td, *J* = 7.5, 1.4 Hz, 1H), 7.37 (dd, *J* = 7.6, 0.9 Hz, 1H), 6.51 (t, *J* = 5.8 Hz, 1H), 5.87 (t, *J* = 6.1 Hz, 1H), 4.89 (td, *J* = 7.9, 5.0 Hz, 1H), 3.79 (s, 3H), 3.50 – 3.40 (m, 4H), 3.18 – 3.11 (m, 2H), 2.28 (dtd, *J* = 14.1, 6.9, 5.0 Hz, 1H), 2.12 (ddt, *J* = 14.3, 7.9, 6.5 Hz, 1H).

¹³C NMR (101 MHz, CDCl₃) δ/ppm: 171.62, 169.34, 161.89, 148.13, 145.35, 140.99, 140.62, 135.96, 134.43, 133.87, 133.12, 133.02, 131.11, 130.98, 130.59, 129.64, 128.70, 125.32, 124.15, 52.85, 50.86, 47.89, 42.95, 39.92, 31.24.

HRMS (ESI): C₂₆H₂₆N₈NaO₈S⁺ *calcd*: 633.1487, *found*: 633.1495.

6.17 Nos-Sc-NP12-TFL2(N₃)-OH **XZ17**



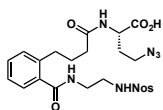
In a 100 mL flask **XZ13** (3.9 g, 6.8 mmol, 1.0 eq.) was dissolved in MeCN (40 mL) and a solution of LiOH·H₂O (1.4 g, 34.0 mmol, 5.0 eq.) in H₂O (20 mL) was added. The mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed full conversion. Acidifying the mixture to pH 5-6 with concentrated HCl quenched the reaction and the volatiles were removed *in vacuo*. The residue was taken up in EtOAc (100 mL) and H₂O (100 mL). The biphasic mixture was acidified to pH 1 with concentrated HCl and the layers were separated. The aqueous layer was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with half-saturated brine (2 x 100 mL) and brine (100 mL), dried over MgSO₄ and concentrated to yield the desired product as a slightly yellow solid (2.8 g, 73%). NMR showed some residual tetramethylurea from the HBTU coupling step. The material was used without further purification for the next synthetic step.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 12.65 (s, 1H), 8.31 (t, *J* = 5.7 Hz, 1H), 8.21 (t, *J* = 5.9 Hz, 1H), 8.06 – 8.01 (m, 1H), 8.01 – 7.94 (m, 2H), 7.91 – 7.83 (m, 2H), 7.37 – 7.28 (m, 2H), 7.27 – 7.17 (m, 2H), 4.23 (ddd, *J* = 9.7, 8.1, 4.4 Hz, 1H), 3.48 – 3.20 (m, 3H), 3.18 – 2.98 (m, 4H), 2.95 – 2.83 (m, 1H), 2.76 – 2.62 (m, 1H), 1.86 (dtd, *J* = 13.9, 7.7, 4.5 Hz, 1H), 1.76 – 1.57 (m, 1H), 0.98 (d, *J* = 6.4 Hz, 3H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 175.30, 173.04, 169.30, 147.72, 137.80, 136.86, 134.07, 132.70, 132.67, 129.93, 129.47, 129.13, 127.32, 125.82, 124.49, 48.94, 47.25, 42.08, 41.26, 36.33, 30.13, 17.83.

HRMS (ESI): $C_{23}H_{27}N_7NaO_8S^+$ *calcd*: 584.1534, *found*: 584.1540.

6.18 Nos-Sc-NP14-TFL2(N₃)-OH XZ18



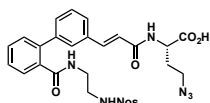
In a 100 mL flask **XZ14** (2.6 g, 4.5 mmol, 1.0 eq.) was dissolved in MeCN (25 mL) and a solution of LiOH·H₂O (943.0 mg, 22.5 mmol, 5.0 eq.) in H₂O (12.5 mL) was added. The mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed full conversion to the desired product. Acidifying the mixture to pH 5-6 with concentrated HCl quenched the reaction and the volatiles were removed *in vacuo*. The residue was taken up in EtOAc (100 mL) and H₂O (100 mL). The biphasic mixture was acidified to pH 1 with concentrated HCl and the layers were separated. The aqueous layer was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with half-saturated brine (2 x 100 mL) and brine (100 mL), dried over MgSO₄ and concentrated by rotary evaporation to yield the desired product as a yellowish solid (2.5 g, **98%**). NMR showed some residual tetramethylurea from the HBTU coupling step. The material was used without further purification for the next synthetic step.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 12.65 (s, 1H), 8.29 (t, *J* = 5.7 Hz, 1H), 8.19 (t, *J* = 5.8 Hz, 1H), 8.11 (d, *J* = 7.9 Hz, 1H), 8.06 – 8.01 (m, 1H), 8.01 – 7.96 (m, 1H), 7.90 – 7.84 (m, 2H), 7.38 – 7.28 (m, 2H), 7.28 – 7.17 (m, 2H), 4.27 (ddd, *J* = 9.6, 7.9, 4.6 Hz, 1H), 3.46 – 3.34 (m, 2H), 3.34 – 3.27 (m, 2H), 3.12 – 3.05 (m, 2H), 2.65 (dd, *J* = 8.8, 6.7 Hz, 2H), 2.11 (t, *J* = 7.5 Hz, 2H), 1.98 – 1.88 (m, 1H), 1.88 – 1.79 (m, 1H), 1.79 – 1.70 (m, 2H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 173.21, 172.21, 169.35, 147.70, 139.64, 136.70, 134.05, 132.71, 132.70, 129.72, 129.46, 129.33, 127.23, 125.59, 124.48, 49.27, 47.57, 42.07, 39.00, 34.89, 32.06, 30.14, 27.12.

HRMS (ESI): $C_{23}H_{27}N_7NaO_8S^+$ *calcd*: 584.1534, *found*: 584.1546.

6.19 Nos-Sc-NP01-TFL2(N₃)-OH XZ19



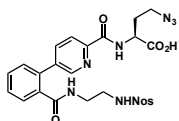
In a 100 mL flask **XZ15** (3.6 g, 5.7 mmol, 1.0 eq.) was dissolved in MeCN (34 mL) and a solution of LiOH·H₂O (1.2 g, 28.5 mmol, 5.0 eq.) in H₂O (17 mL) was added. The mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed full conversion to the desired product. Acidifying the mixture to pH 5-6 with concentrated HCl quenched the reaction and the volatiles were removed *in vacuo*. The residue was taken up in EtOAc (100 mL) and H₂O (100 mL). The biphasic mixture was acidified to pH 1 with concentrated HCl and the layers were separated. The aqueous layer was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with half-saturated brine (2 x 100 mL) and brine (100 mL), dried over MgSO₄ and concentrated by rotary evaporation to yield the desired product as a yellowish solid (2.8 g, **79%**). NMR showed some residual tetramethylurea from the HBTU coupling step. The material was used without further purification for the next synthetic step.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 12.81 (s, 1H), 8.46 (d, *J* = 7.9 Hz, 1H), 8.18 (t, *J* = 5.8 Hz, 1H), 8.09 (t, *J* = 6.0 Hz, 1H), 8.02 – 7.97 (m, 1H), 7.96 – 7.92 (m, 1H), 7.92 – 7.87 (m, 2H), 7.55 – 7.49 (m, 2H), 7.48 – 7.40 (m, 5H), 7.37 – 7.29 (m, 2H), 6.73 (d, *J* = 15.8 Hz, 1H), 4.44 (ddd, *J* = 9.2, 7.9, 4.8 Hz, 1H), 3.51 – 3.38 (m, 2H), 3.14 (dt, *J* = 7.9, 6.1 Hz, 2H), 2.79 – 2.72 (m, 2H), 2.05 (dtd, *J* = 13.9, 7.4, 4.8 Hz, 1H), 1.95 – 1.85 (m, 1H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 173.06, 169.12, 165.00, 147.70, 140.79, 139.12, 138.57, 136.90, 134.63, 134.10, 132.73, 132.48, 129.72, 129.56, 129.52, 129.39, 128.62, 127.65, 127.60, 127.33, 126.27, 124.54, 121.81, 49.63, 47.53, 41.56, 38.75, 30.40.

HRMS (ESI): $C_{28}H_{27}N_7NaO_8S^+$ *calcd*: 644.1534, *found*: 644.1537.

6.20 Nos-Sc-NP07-TFL2(N₃)-OH XZ20



In a 50 mL flask **XZ16** (367 mg, 601.0 μmol, 1.0 eq.) was dissolved in MeCN (5 mL) and a solution of LiOH·H₂O (126 mg, 3.0 mmol, 5.0 eq.) in H₂O (2.5 mL) was added. The mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed full conversion to the desired product. Acidifying the mixture to pH 5-6 with concentrated HCl quenched the reaction and the volatiles were removed *in vacuo*. The residue was taken up in EtOAc (50 mL) and H₂O (50 mL). The biphasic mixture was acidified to pH 1 with concentrated

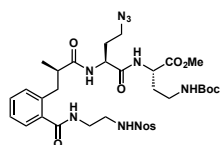
HCl and the layers were separated. The aqueous layer was extracted with EtOAc (2 x 50 mL) and the combined organic layers were washed with half-saturated brine (2 x 50 mL) and brine (50 mL), dried over MgSO₄ and concentrated by rotary evaporation to yield the desired product as a thick, waxy oil (400 mg, >100%). NMR showed some residual tetramethylurea from the HBTU coupling step. The material was used without further purification for the next synthetic step.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 12.92 (s, 1H), 8.98 (d, *J* = 8.3 Hz, 1H), 8.62 (dd, *J* = 2.2, 0.9 Hz, 1H), 8.47 (t, *J* = 5.7 Hz, 1H), 8.15 (t, *J* = 5.9 Hz, 1H), 8.02 (dd, *J* = 8.1, 0.8 Hz, 1H), 8.00 – 7.96 (m, 2H), 7.93 (dd, *J* = 8.1, 2.2 Hz, 1H), 7.91 – 7.81 (m, 2H), 7.65 – 7.46 (m, 4H), 4.58 (dt, *J* = 8.2, 6.9 Hz, 1H), 3.48 (dt, *J* = 12.4, 6.2 Hz, 1H), 3.39 (dt, *J* = 12.5, 7.2 Hz, 1H), 3.19 (q, *J* = 6.4 Hz, 2H), 2.94 (q, *J* = 6.5 Hz, 2H), 2.15 (q, *J* = 6.8 Hz, 2H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 172.76, 168.56, 163.86, 147.94, 147.66, 147.57, 138.80, 137.16, 136.72, 135.31, 134.10, 132.71, 132.57, 130.18, 130.01, 129.42, 128.29, 128.10, 124.49, 121.46, 49.77, 47.76, 41.81, 38.98, 30.03.

HRMS (ESI): C₂₅H₂₄N₈NaO₈S⁺ *calcd*: 619.1330, *found*: 619.1332.

6.21 Nos-Sc-NP12-TFL2(N₃)-AA070-OMe XZ21



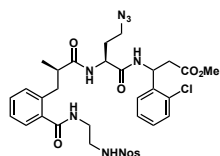
In a 50 mL flask **XZ17** (1.3 g, 2.3 mmol, 1.0 eq.) was dissolved in THF (20 mL) and H-Dab(Boc)-OMe hydrochloride (747.0 mg, 2.8 mmol, 1.2 eq.) was added, followed by DIPEA (1.2 mL, 6.9 mmol, 3.0 eq.) and HATU (1.1 g, 2.8 mmol, 1.2 eq.). The mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was taken up in EtOAc (100 mL) and half-saturated NH₄Cl solution (100 mL). The biphasic mixture was separated and the aqueous layer was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with saturated NH₄Cl solution (2 x 100 mL), brine (100 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 120 g, DCM/MeOH, UV) to yield the desired product as a slightly yellow solid (1.8 g, >100%). NMR showed some residual tetramethylurea from the coupling step. The material was used without further purification for the next synthetic transformation.

¹H NMR (400 MHz, Acetonitrile-*d*₃) δ/ppm: 8.11 – 8.04 (m, 1H), 7.88 – 7.82 (m, 1H), 7.82 – 7.77 (m, 2H), 7.41 – 7.31 (m, 3H), 7.31 – 7.20 (m, 2H), 7.06 (d, *J* = 8.5 Hz, 1H), 6.93 (d, *J* = 8.1 Hz, 1H), 6.89 – 6.81 (m, 1H), 5.40 (s, 1H), 4.38 (ddd, *J* = 9.1, 8.0, 4.8 Hz, 1H), 4.30 (ddd, *J* = 9.0, 8.0, 4.8 Hz, 1H), 3.64 (s, 3H), 3.63 – 3.51 (m, 1H), 3.42 – 3.34 (m, 1H), 3.33 – 3.12 (m, 3H), 3.11 – 3.02 (m, 2H), 3.01 – 2.84 (m, 3H), 2.82 – 2.76 (m, 2H), 1.82 (dtd, *J* = 14.0, 7.6, 4.9 Hz, 1H), 1.70 (ddt, *J* = 14.7, 9.1, 6.0 Hz, 1H), 1.57 (dddd, *J* = 14.4, 9.0, 7.1, 5.6 Hz, 1H), 1.38 (s, 9H), 1.14 (d, *J* = 6.5 Hz, 3H).

¹³C NMR (101 MHz, CD₃CN) δ/ppm: 177.24, 173.07, 172.00, 171.26, 156.86, 148.97, 138.68, 137.59, 135.00, 133.97, 133.79, 131.40, 131.30, 130.70, 128.48, 127.28, 125.84, 80.09, 53.61, 52.89, 51.25, 50.77, 48.16, 44.01, 43.47, 40.60, 37.68, 32.43, 31.66, 28.60, 18.36.

HRMS (ESI): C₃₃H₄₅N₆NaO₁₁S⁺ *calcd*: 798.2851, *found*: 798.2862.

6.22 Nos-Sc-NP12-TFL2(N₃)-AA118-OMe XZ22



In a 25 mL flask **XZ17** (350.0 mg, 623.0 μmol, 1.0 eq.) was dissolved in THF (5 mL) and methyl 3-amino-3-(2-chlorophenyl)propanoate (160.0 mg, 748.0 μmol, 1.2 eq.) was added, followed by DIPEA (326.0 μL, 1.9 mmol, 3.0 eq.) and HATU (284 mg, 746.0 μmol, 1.2 eq.). The mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was taken up in EtOAc (50 mL) and H₂O (50 mL). The mixture was acidified with concentrated HCl to pH 1. The layers were separated and the aqueous phase was extracted with EtOAc (2 x 50 mL) and the combined organic layers were washed with diluted HCl solution (1%, 2 x 50 mL), brine (50 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 120 g, DCM/MeOH, UV) to yield the desired product as a slightly yellow solid (403 mg, 85%). NMR and UPLC-MS analysis showed a mixture of four diastereomers in the ratio 7:36:42:15.

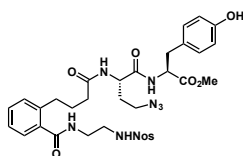
¹H NMR (500 MHz, Acetonitrile-*d*₃) δ/ppm: 8.12 – 7.99 (m, 1H), 7.91 – 7.80 (m, 1H), 7.80 – 7.70 (m, 2H), 7.40 – 7.34 (m, 3H), 7.34 – 7.13 (m, 7H), 6.96 (t, *J* = 6.0 Hz, 1H), 6.86 (d, *J* = 8.1 Hz, 1H), 5.58 – 5.44 (m, 1H), 4.42 – 4.14 (m, 1H), 3.59 – 3.55 (m, 3H), 3.49 –

3.11 (m, 4H), 3.12 – 3.03 (m, 1H), 2.90 (dddd, $J = 14.5, 12.7, 7.3, 5.4$ Hz, 1H), 2.83 – 2.73 (m, 3H), 2.72 – 2.56 (m, 2H), 1.83 – 1.67 (m, 1H), 1.61 – 1.44 (m, 1H), 1.18 (d, $J = 6.6$ Hz, 3H).

^{13}C NMR (101 MHz, CD_3CN) δ /ppm: 177.21, 171.70, 171.23, 166.26, 149.09, 139.60, 139.45, 138.74, 137.70, 137.56, 134.93, 132.74, 131.45, 131.24, 131.13, 130.54, 129.82, 128.52, 128.42, 128.31, 127.28, 125.75, 52.29, 51.25, 48.45, 48.08, 43.80, 43.43, 40.56, 39.37, 37.52, 31.36, 18.33.

HRMS (ESI): $\text{C}_{33}\text{H}_{37}\text{ClN}_8\text{NaO}_9\text{S}^+$ *calcd*: 779.1985, *found*: 779.1992.

6.23 Nos-Sc-NP14-TFL2(N_3)-AA020-OMe XZ23



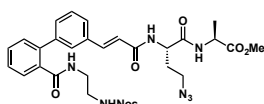
In a 50 mL flask **XZ18** (1.3 g, 2.3 mmol, 1.0 eq.) was dissolved in THF (20 mL) and H-Tyr-OMe (542.0 mg, 2.8 mmol, 1.2 eq.) was added, followed by DIPEA (1.2 mL, 6.9 mmol, 3.0 eq.) and HATU (1.1 g, 2.8 mmol, 1.2 eq.). The mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was taken up in EtOAc (100 mL) and H_2O (100 mL). The mixture was acidified with concentrated HCl to pH 1. The layers were separated and the aqueous phase was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with diluted HCl solution (1%, 2 x 100 mL), brine (100 mL), dried over Na_2SO_4 and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 120 g, DCM/MeOH, UV) to yield the desired product as a slightly yellow solid (1.1 g, **66%**).

^1H NMR (400 MHz, Acetonitrile- d_3) δ /ppm: 8.11 – 8.02 (m, 1H), 7.88 – 7.81 (m, 1H), 7.81 – 7.73 (m, 2H), 7.39 – 7.31 (m, 2H), 7.28 – 7.20 (m, 2H), 7.04 (t, $J = 6.0$ Hz, 1H), 7.00 – 6.89 (m, 3H), 6.76 (d, $J = 8.0$ Hz, 1H), 6.72 – 6.64 (m, 2H), 6.51 (t, $J = 5.8$ Hz, 1H), 4.56 (td, $J = 7.7, 5.3$ Hz, 1H), 4.36 (td, $J = 8.4, 5.5$ Hz, 1H), 3.64 (s, 3H), 3.53 – 3.41 (m, 2H), 3.38 – 3.27 (m, 2H), 3.27 – 3.21 (m, 2H), 3.00 (dd, $J = 14.0, 5.3$ Hz, 1H), 2.86 (dd, $J = 14.0, 7.7$ Hz, 1H), 2.78 – 2.65 (m, 2H), 2.17 (dt, $J = 7.2, 3.5$ Hz, 2H), 2.01 – 1.91 (m, 1H), 1.86 – 1.69 (m, 3H).

^{13}C NMR (101 MHz, CD_3CN) δ /ppm: 173.98, 172.67, 171.86, 171.76, 156.84, 148.96, 140.91, 137.25, 137.16, 135.04, 133.83, 131.34, 131.32, 131.11, 130.87, 128.42, 128.08, 126.89, 125.91, 116.07, 54.61, 52.74, 51.42, 48.78, 43.97, 40.36, 37.07, 36.15, 33.30, 31.38, 28.05.

HRMS (ESI): $\text{C}_{33}\text{H}_{38}\text{N}_8\text{NaO}_{10}\text{S}^+$ *calcd*: 761.2324, *found*: 761.2320.

6.24 Nos-Sc-NP01-TFL2(N_3)-AA001-OMe XZ24



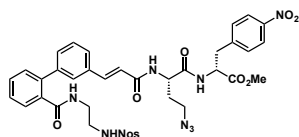
In a 50 mL flask **XZ19** (1.1 g, 1.8 mmol, 1.0 eq.) was dissolved in THF (16 mL) and H-Ala-OMe hydrochloride (298.0 mg, 2.1 mmol, 1.2 eq.) was added, followed by DIPEA (931.0 μL , 5.3 mmol, 3.0 eq.) and HATU (810.0 mg, 2.1 mmol, 1.2 eq.). The mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was taken up in EtOAc (100 mL) and H_2O (100 mL). The mixture was acidified with concentrated HCl to pH 1. The layers were separated and the aqueous phase was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with diluted HCl solution (1%, 2 x 100 mL), brine (100 mL), dried over Na_2SO_4 and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 120 g, DCM/MeOH, UV) to yield the desired product as a slightly yellow solid (1.0 g, **81%**).

^1H NMR (400 MHz, Acetonitrile- d_3) δ /ppm: 8.02 – 7.95 (m, 1H), 7.88 – 7.83 (m, 1H), 7.83 – 7.78 (m, 2H), 7.58 – 7.51 (m, 2H), 7.51 – 7.45 (m, 3H), 7.44 – 7.38 (m, 2H), 7.37 – 7.33 (m, 2H), 7.19 (d, $J = 7.0$ Hz, 1H), 7.05 (d, $J = 8.0$ Hz, 1H), 6.70 – 6.55 (m, 2H), 6.12 (t, $J = 5.9$ Hz, 1H), 4.58 (td, $J = 8.2, 5.4$ Hz, 1H), 4.42 – 4.32 (m, 1H), 3.66 (s, 3H), 3.42 (ddd, $J = 9.9, 5.0, 2.4$ Hz, 2H), 3.22 (q, $J = 6.2$ Hz, 2H), 2.93 (q, $J = 6.1$ Hz, 2H), 2.05 (ddd, $J = 14.3, 7.1, 5.5$ Hz, 1H), 1.92 – 1.83 (m, 1H), 1.32 (d, $J = 7.3$ Hz, 3H).

^{13}C NMR (101 MHz, CD_3CN) δ /ppm: 173.90, 171.89, 170.91, 166.40, 148.89, 142.01, 140.90, 140.03, 137.31, 135.94, 135.11, 133.87, 133.65, 131.40, 131.01, 130.93, 130.88, 129.79, 128.81, 128.76, 128.55, 127.87, 126.02, 122.39, 51.64, 49.14, 48.67, 43.67, 40.08, 38.82, 32.29, 17.53.

HRMS (ESI): $\text{C}_{32}\text{H}_{34}\text{N}_8\text{NaO}_9\text{S}^+$ *calcd*: 729.2062, *found*: 729.2060.

6.25 Nos-Sc-NP01-TFL2(N₃)-AA058-OMe XZ25



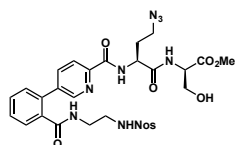
In a 50 mL flask **XZ19** (1.1 g, 1.8 mmol, 1.0 eq.) was dissolved in THF (16 mL) and H-*p*-Nitro-*D*-Phe-OMe hydrochloride (560.0 mg, 2.2 mmol, 1.2 eq.) was added, followed by DIPEA (936.0 μ L, 5.4 mmol, 3.0 eq.) and HATU (815.0 mg, 2.1 mmol, 1.2 eq.). The mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was taken up in EtOAc (100 mL) and H₂O (100 mL). The mixture was acidified with concentrated HCl to pH 1. The layers were separated and the aqueous phase was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with diluted HCl solution (1%, 2 x 100 mL), brine (100 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 120 g, DCM/MeOH, UV) to yield the desired product as a slightly yellow solid (1.3 g, **88%**).

¹H NMR (400 MHz, Acetonitrile-*d*₃) δ /ppm: 8.14 – 8.03 (m, 2H), 8.01 – 7.94 (m, 1H), 7.92 – 7.73 (m, 3H), 7.64 – 7.54 (m, 1H), 7.54 – 7.44 (m, 4H), 7.44 – 7.37 (m, 4H), 7.38 – 7.31 (m, 2H), 7.19 (d, *J* = 8.2 Hz, 1H), 6.97 (d, *J* = 7.8 Hz, 1H), 6.63 (t, *J* = 6.1 Hz, 1H), 6.58 (d, *J* = 15.7 Hz, 1H), 6.11 (t, *J* = 5.8 Hz, 1H), 4.78 – 4.68 (m, 1H), 4.47 (dtd, *J* = 13.4, 8.3, 5.3 Hz, 1H), 3.66 (s, 3H), 3.38 – 3.18 (m, 5H), 3.09 (dt, *J* = 13.9, 8.6 Hz, 1H), 3.01 – 2.88 (m, 2H), 2.03 – 1.85 (m, 1H), 1.85 – 1.68 (m, 1H).

¹³C NMR (101 MHz, CD₃CN) δ /ppm: 172.12, 171.96, 170.93, 166.48, 147.93, 145.95, 142.00, 141.08, 140.02, 137.29, 135.88, 135.11, 133.86, 133.65, 131.47, 131.46, 131.40, 131.02, 130.96, 130.94, 129.80, 128.85, 128.78, 128.57, 127.91, 126.02, 124.33, 122.16, 53.85, 53.02, 51.95, 48.62, 43.70, 40.09, 37.59, 31.78.

HRMS (ESI): C₃₈H₃₇N₉NaO₁₁S⁺ *calcd*: 850.2225, *found*: 850.2225.

6.26 Nos-Sc-NP07-TFL2(N₃)-AA026-OMe XZ26



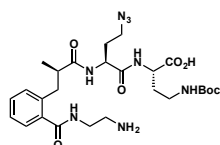
In a 50 mL flask **XZ20** (345.0 mg, 578.0 μ mol, 1.0 eq.) was dissolved in THF (5 mL) and H-*D*-Ser-OMe hydrochloride (108.0 mg, 694.0 μ mol, 1.2 eq.) was added, followed by DIPEA (302.0 μ L, 1.7 mmol, 3.0 eq.) and HATU (263.0 mg, 692.0 μ mol, 1.2 eq.). The mixture was stirred at RT for 1 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed *in vacuo* and the residue was taken up in EtOAc (50 mL) and H₂O (50 mL). The mixture was acidified with concentrated HCl to pH 1. The layers were separated and the aqueous phase was extracted with EtOAc (2 x 100 mL) and the combined organic layers were washed with diluted HCl solution (1%, 2 x 50 mL), brine (50 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by flash column chromatography on the ISOLERA (Silica, 120 g, DCM/MeOH, UV) to yield the desired product as a waxy solid (403.0 mg, **94%**).

¹H NMR (500 MHz, Acetonitrile-*d*₃) δ /ppm: 8.62 (dd, *J* = 2.3, 0.8 Hz, 1H), 8.57 (d, *J* = 8.2 Hz, 1H), 8.08 (dd, *J* = 8.0, 0.8 Hz, 1H), 8.05 – 8.00 (m, 1H), 7.92 (dd, *J* = 8.1, 2.2 Hz, 1H), 7.89 – 7.84 (m, 1H), 7.81 (dd, *J* = 5.9, 3.4 Hz, 2H), 7.61 – 7.55 (m, 2H), 7.53 – 7.49 (m, 1H), 7.48 (ddd, *J* = 7.5, 1.4, 0.6 Hz, 1H), 7.25 (d, *J* = 7.8 Hz, 1H), 6.89 (t, *J* = 6.0 Hz, 1H), 6.12 (s, 1H), 4.74 (td, *J* = 8.3, 5.1 Hz, 1H), 4.48 (dt, *J* = 8.1, 4.1 Hz, 1H), 3.89 – 3.82 (m, 1H), 3.76 (ddd, *J* = 11.2, 5.5, 3.8 Hz, 1H), 3.66 (s, 3H), 3.46 (ddd, *J* = 7.5, 6.2, 3.1 Hz, 2H), 3.29 (q, *J* = 6.0 Hz, 2H), 3.22 (t, *J* = 6.0 Hz, 1H), 3.08 (q, *J* = 5.7 Hz, 2H), 2.19 (ddd, *J* = 14.5, 7.3, 2.2 Hz, 1H), 2.05 (ddt, *J* = 14.5, 8.4, 6.3 Hz, 1H).

¹³C NMR (126 MHz, CD₃CN) δ /ppm: 171.88, 171.56, 170.37, 164.98, 148.92, 140.13, 138.27, 137.39, 136.61, 135.13, 133.82, 133.67, 131.38, 131.35, 131.29, 129.55, 129.52, 128.99, 125.99, 122.42, 62.49, 55.69, 52.79, 51.85, 48.86, 43.76, 40.06, 32.30.

HRMS (ESI): C₂₉H₃₁N₉NaO₁₀S⁺ *calcd*: 720.1807, *found*: 720.1813.

6.27 NH₂-Sc-NP12-TFL2(N₃)-AA070-OH XZ27



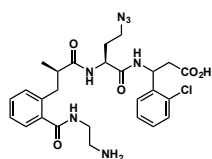
Under an inert (N₂) atmosphere **XZ21** (1.8 g, 2.3 mmol, 1.0 eq.) was dissolved in MeCN (24 mL) and DIPEA (4.0 mL, 22.8 mmol, 10.0 eq.) was added, followed by thiophenol (1.2 mL, 11.4 mmol, 5.0 eq.). The yellow solution was stirred at RT for 2 h after which UPLC-MS analysis showed complete Nosyl deprotection. A solution of LiOH·H₂O (764.0 mg, 18.2 mmol, 8.0 eq.) in H₂O (8 mL) was added and the mixture was stirred for 1.5 h after which UPLC-MS analysis showed complete ester deprotection. The volatiles were removed by rotary evaporation and the crude was purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, H₂O/MeCN, UV (200/215 nm)). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (999 mg, **76%**). NMR and LC-MS analysis showed 19% epimerized product.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 8.55 (t, *J* = 5.9 Hz, 1H), 8.25 (d, *J* = 8.1 Hz, 1H), 7.66 (d, *J* = 6.9 Hz, 1H), 7.48 – 7.26 (m, 2H), 7.27 – 7.02 (m, 2H), 6.66 (t, *J* = 5.7 Hz, 1H), 4.16 (td, *J* = 8.8, 8.2, 4.2 Hz, 1H), 3.73 (q, *J* = 6.5 Hz, 1H), 3.50 – 3.34 (m, 1H), 3.37 – 3.20 (m, 1H), 3.15 – 2.52 (m, 9H), 2.03 – 1.61 (m, 2H), 1.63 – 1.39 (m, 2H), 1.35 (s, 9H), 1.04 (d, *J* = 6.2 Hz, 3H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 175.43, 173.73, 169.99, 169.43, 155.34, 137.73, 137.19, 130.05, 129.03, 127.24, 125.79, 77.34, 52.51, 50.27, 47.31, 41.60, 39.80, 39.51, 37.31, 36.28, 33.22, 30.68, 28.26, 17.95.

HRMS (ESI): C₂₆H₄₁N₈O₇⁺ *calcd*: 577.3093, *found*: 577.3101.

6.28 NH₂-Sc-NP12-TFL2(N₃)-AA118-OH **XZ28**



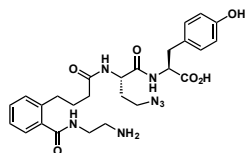
Under an inert (N₂) atmosphere **XZ22** (406.0 mg, 536.0 μmol, 1.0 eq.) was dissolved in MeCN (5 mL) and DIPEA (934.0 μL, 5.4 mmol, 10.0 eq.) was added, followed by thiophenol (273.0 μL, 2.7 mmol, 5.0 eq.). The yellow solution was stirred at RT for 2 h after which UPLC-MS analysis showed complete Nosyl deprotection. A solution of LiOH·H₂O (171.0 mg, 4.1 mmol, 8.0 eq.) in H₂O (1.5 mL) was added and the mixture was stirred for 1.5 h after which UPLC-MS analysis showed complete ester deprotection. The mixture was acidified to pH 5-6 by the addition of concentrated HCl. The volatiles were removed by rotary evaporation and the crude was purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 120 g, H₂O/MeCN + 0.1% TFA, UV (200/215 nm)). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (331 mg, **>100%**). Co-elution of unknown sideproducts. NMR and LC-MS analysis showed a diastereomeric ratio of 9:36:43:12.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 8.56 (d, *J* = 7.7 Hz, 1H), 8.39 (t, *J* = 5.4 Hz, 1H), 8.17 – 7.58 (m, 4H), 7.57 – 6.82 (m, 8H), 5.48 (dd, *J* = 8.0, 6.3 Hz, 1H), 4.38 – 4.21 (m, 1H), 3.58 – 3.17 (m, 2H), 3.09 – 2.81 (m, 4H), 2.80 – 2.54 (m, 4H), 2.48 – 2.33 (m, 1H), 1.80 – 1.44 (m, 2H), 0.96 (d, *J* = 6.3 Hz, 3H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 171.41, 171.35, 170.06, 169.71, 139.51, 139.48, 137.97, 136.41, 131.70, 131.50, 130.07, 129.41, 128.80, 127.47, 127.34, 125.84, 49.78, 47.17, 46.98, 41.23, 39.22, 38.63, 36.91, 36.48, 31.14, 17.76.

HRMS (ESI): C₂₆H₃₃ClN₇O₅⁺ *calcd*: 558.2226, *found*: 558.2218.

6.29 NH₂-Sc-NP14-TFL2(N₃)-AA020-OH **XZ29**



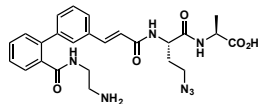
Under an inert (N₂) atmosphere **XZ23** (1.1 g, 1.5 mmol, 1.0 eq.) was dissolved in MeCN (15 mL) and DIPEA (2.6 mL, 14.8 mmol, 10.0 eq.) was added, followed by thiophenol (757.0 μL, 7.4 mmol, 5.0 eq.). The yellow solution was stirred at RT for 2 h after which UPLC-MS analysis showed complete Nosyl deprotection. A solution of LiOH·H₂O (489.0 mg, 11.7 mmol, 8.0 eq.) in H₂O (5 mL) was added and the mixture was stirred for 1.5 h after which UPLC-MS analysis showed complete ester deprotection. The mixture was acidified to pH 5-6 by the addition of concentrated HCl. The volatiles were removed by rotary evaporation and the crude was purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, H₂O/MeCN + 0.1% TFA, UV (200/215 nm)). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (1.0 g, **>100%**). Co-elution of unknown sideproducts. NMR and LC-MS analysis showed 16% epimerized product.

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 8.39 (t, *J* = 5.6 Hz, 1H), 8.07 (d, *J* = 7.7 Hz, 1H), 7.99 (d, *J* = 8.2 Hz, 1H), 7.90 (brs, 3H), 7.43 (dd, *J* = 7.9, 1.5 Hz, 1H), 7.37 (td, *J* = 7.5, 1.5 Hz, 1H), 7.25 (dtd, *J* = 7.5, 3.8, 1.3 Hz, 2H), 7.05 – 6.93 (m, 2H), 6.69 – 6.57 (m, 2H), 4.34 (dtd, *J* = 16.0, 8.4, 5.2 Hz, 2H), 3.46 (q, *J* = 6.2 Hz, 2H), 3.37 – 3.18 (m, 2H), 2.98 (q, *J* = 6.0 Hz, 2H), 2.92 (dd, *J* = 14.0, 5.1 Hz, 1H), 2.79 (dd, *J* = 13.9, 8.5 Hz, 1H), 2.68 (t, *J* = 7.8 Hz, 2H), 2.20 – 2.02 (m, 2H), 1.88 (dtd, *J* = 13.1, 7.6, 4.7 Hz, 1H), 1.81 – 1.66 (m, 3H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 172.83, 172.03, 171.09, 169.77, 155.97, 139.86, 136.22, 130.04, 129.87, 129.61, 127.46, 127.33, 125.62, 114.98, 53.80, 49.79, 47.55, 38.56, 36.94, 35.75, 34.96, 32.20, 31.14, 27.12.

HRMS (ESI): $\text{C}_{26}\text{H}_{34}\text{N}_7\text{O}_6^+$ *calcd*: 540.2565, *found*: 540.2560.

6.30 NH_2 -Sc-NP01-TFL2(N_3)-AA001-OH XZ30



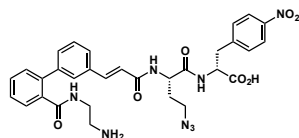
Under an inert (N_2) atmosphere **XZ24** (1.0 g, 1.4 mmol, 1.0 eq.) was dissolved in MeCN (12 mL) and DIPEA (2.5 mL, 14.2 mmol, 10.0 eq.) was added, followed by thiophenol (725.0 μL , 7.1 mmol, 5.0 eq.). The yellow solution was stirred at RT for 2 h after which UPLC-MS analysis showed complete Nosyl deprotection. A solution of $\text{LiOH}\cdot\text{H}_2\text{O}$ (468.0 mg, 11.2 mmol, 8.0 eq.) in H_2O (4 mL) was added and the mixture was stirred for 1.5 h after which UPLC-MS analysis showed complete ester deprotection. The mixture was acidified to pH 5-6 by the addition of concentrated HCl. The volatiles were removed by rotary evaporation and the crude was purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, $\text{H}_2\text{O}/\text{MeCN}$ + 0.1% TFA, UV (254/280 nm)). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (810 mg, **>100%**). Co-elution of unknown sideproducts. NMR and LC-MS analysis showed 18% epimerized product.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.44 (d, J = 7.1 Hz, 1H), 8.38 – 8.28 (m, 2H), 7.81 (brs, 3H), 7.59 – 7.51 (m, 4H), 7.49 – 7.41 (m, 4H), 7.37 (dt, J = 7.6, 1.5 Hz, 1H), 6.81 (d, J = 15.8 Hz, 1H), 4.54 (td, J = 8.2, 5.2 Hz, 1H), 4.27 – 4.16 (m, 1H), 3.40 (tdd, J = 10.1, 8.8, 5.7 Hz, 2H), 3.28 (q, J = 6.5 Hz, 2H), 2.74 (q, J = 6.3 Hz, 2H), 2.02 – 1.90 (m, 1H), 1.83 (dtd, J = 13.8, 7.9, 6.1 Hz, 1H), 1.30 (d, J = 7.2 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 173.94, 170.77, 169.58, 164.77, 140.82, 138.69, 136.50, 134.82, 132.39, 129.84, 129.76, 129.49, 128.77, 127.87, 127.64, 127.36, 126.33, 122.24, 49.97, 47.57, 47.45, 38.11, 36.74, 31.59, 16.89.

HRMS (ESI): $\text{C}_{25}\text{H}_{30}\text{N}_7\text{O}_5^+$ *calcd*: 508.2303, *found*: 508.2297.

6.31 NH_2 -Sc-NP01-TFL2(N_3)-AA058-OH XZ31



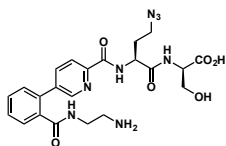
Under an inert (N_2) atmosphere **XZ25** (1.3 g, 1.6 mmol, 1.0 eq.) was dissolved in MeCN (15 mL) and DIPEA (2.7 mL, 15.5 mmol, 10.0 eq.) was added, followed by thiophenol (788.0 μL , 7.7 mmol, 5.0 eq.). The yellow solution was stirred at RT for 2 h after which UPLC-MS analysis showed complete Nosyl deprotection. A solution of $\text{LiOH}\cdot\text{H}_2\text{O}$ (511.0 mg, 12.2 mmol, 8.0 eq.) in H_2O (5 mL) was added and the mixture was stirred for 1.5 h after which UPLC-MS analysis showed complete ester deprotection. The mixture was acidified to pH 5-6 by the addition of concentrated HCl. The volatiles were removed by rotary evaporation and the crude was purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, $\text{H}_2\text{O}/\text{MeCN}$ + 0.1% TFA, UV (254/280 nm)). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (940 mg, **97%**). NMR and LC-MS analysis showed 25% epimerized product.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.55 (d, J = 8.5 Hz, 1H), 8.33 (t, J = 5.6 Hz, 1H), 8.28 (dd, J = 8.3, 2.9 Hz, 1H), 8.13 (dd, J = 8.8, 7.1 Hz, 2H), 7.80 (brs, 3H), 7.64 – 7.50 (m, 6H), 7.50 – 7.41 (m, 4H), 7.37 (dt, J = 7.6, 1.5 Hz, 1H), 6.79 (d, J = 15.9 Hz, 1H), 4.56 (dddd, J = 10.3, 8.0, 6.4, 3.7 Hz, 1H), 4.48 (td, J = 8.7, 5.5 Hz, 1H), 3.37 – 3.10 (m, 4H), 3.10 – 2.99 (m, 2H), 2.80 – 2.62 (m, 2H), 1.95 – 1.68 (m, 1H), 1.66 – 1.50 (m, 1H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 172.29, 170.72, 169.57, 164.70, 146.29, 146.00, 140.82, 138.97, 138.68, 136.49, 134.79, 130.64, 130.57, 129.83, 129.75, 128.75, 127.86, 127.63, 127.36, 123.18, 123.13, 122.15, 52.65, 50.04, 47.22, 38.10, 36.73, 36.47, 31.45.

HRMS (ESI): $\text{C}_{31}\text{H}_{33}\text{N}_6\text{O}_7^+$ *calcd*: 629.2467, *found*: 629.2463.

6.32 NH_2 -Sc-NP07-TFL2(N_3)-AA026-OH XZ32



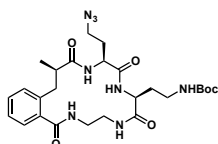
Under an inert (N_2) atmosphere **XZ26** (374.0 mg, 536.0 μ mol, 1.0 eq.) was dissolved in MeCN (5 mL) and DIPEA (934.0 μ L, 5.4 mmol, 10.0 eq.) was added, followed by thiophenol (273.0 μ L, 2.7 mmol, 5.0 eq.). The yellow solution was stirred at RT for 2 h after which UPLC-MS analysis showed complete Nosyl deprotection. A solution of LiOH \cdot H $_2$ O (170.0 mg, 4.1 mmol, 8.0 eq.) in H $_2$ O (1.5 mL) was added and the mixture was stirred for 1.5 h after which UPLC-MS analysis showed complete ester deprotection. The mixture was acidified to pH 5-6 by the addition of concentrated HCl. The volatiles were removed by rotary evaporation and the crude was purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 120 g, H $_2$ O/MeCN + 0.1% TFA, UV (254/280 nm)). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (292 mg, >100%). Co-elution of unknown sideproducts. NMR and LC-MS analysis showed 19% epimerized product.

1H NMR (400 MHz, DMSO- d_6) δ /ppm: 8.85 (d, J = 8.6 Hz, 1H), 8.65 (dd, J = 2.3, 0.9 Hz, 1H), 8.56 (t, J = 5.6 Hz, 1H), 8.47 (d, J = 7.9 Hz, 1H), 8.08 (dd, J = 8.1, 0.8 Hz, 1H), 7.97 (dd, J = 8.1, 2.2 Hz, 1H), 7.80 (brs, 3H), 7.67 (dd, J = 7.5, 1.5 Hz, 1H), 7.62 (td, J = 7.5, 1.5 Hz, 1H), 7.60 – 7.49 (m, 2H), 4.77 (td, J = 8.4, 5.1 Hz, 1H), 4.31 (ddd, J = 7.8, 5.3, 4.1 Hz, 1H), 3.72 (dd, J = 10.8, 5.4 Hz, 1H), 3.66 (dd, J = 10.9, 4.2 Hz, 1H), 3.49 – 3.26 (m, 4H), 2.92 – 2.76 (m, 2H), 2.06 (dddd, J = 19.8, 13.7, 9.2, 5.5 Hz, 2H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 171.61, 170.66, 168.93, 163.24, 147.86, 147.68, 138.89, 137.28, 136.28, 135.45, 132.35, 130.31, 130.26, 128.24, 121.48, 61.17, 54.78, 50.37, 47.60, 38.22, 36.88, 31.99.

HRMS (ESI): $C_{22}H_{27}N_6O_6^+$ *calcd*: 499.2048, *found*: 499.2041.

6.33 MC NP12/AA070 XZ33



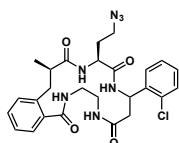
In a 1L flask **XZ27** (955.0 mg, 1.7 mmol, 1.0 eq.) was dissolved in THF (600 mL) and DIPEA (865.0 μ L, 5.0 mmol, 3.0 eq.) was added, followed by HATU (1.1 g, 3.0 mmol, 1.8 eq.). The mixture was stirred at RT for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The mixture was concentrated by rotary evaporation and purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, H $_2$ O:MeCN, UV 200/215 nm) to yield the desired product as a white solid (370.0 mg, 40%). 89 mg of the product were purified again by preparative RP-HPLC (Method A, no TFA) for final analytics and protein binding assays. Mixture of diastereomers with the ratio 10:85:5 (UPLC-MS analysis).

1H NMR (500 MHz, DMSO- d_6) δ /ppm: 8.20 (d, J = 5.4 Hz, 1H), 8.09 (t, J = 5.4 Hz, 1H), 7.74 – 7.68 (m, 1H), 7.66 (d, J = 8.0 Hz, 1H), 7.36 (td, J = 7.4, 1.6 Hz, 1H), 7.29 (d, J = 7.2 Hz, 1H), 7.27 (dd, J = 7.6, 1.6 Hz, 1H), 7.22 (td, J = 7.4, 1.3 Hz, 1H), 6.76 (t, J = 5.5 Hz, 1H), 4.05 (ddd, J = 10.2, 7.9, 4.3 Hz, 1H), 3.72 (dt, J = 8.5, 5.8 Hz, 1H), 3.53 – 3.46 (m, 1H), 3.46 – 3.37 (m, 2H), 3.29 – 3.19 (m, 3H), 3.16 – 3.09 (m, 1H), 2.98 – 2.87 (m, 2H), 2.72 – 2.62 (m, 1H), 2.56 (dd, J = 13.9, 7.1 Hz, 1H), 2.06 – 1.99 (m, 1H), 1.99 – 1.89 (m, 2H), 1.78 – 1.69 (m, 1H), 1.36 (s, 9H), 1.18 (d, J = 7.0 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 176.63, 171.27, 171.08, 169.56, 155.53, 137.72, 137.34, 129.51, 129.09, 127.47, 125.84, 77.52, 53.44, 51.25, 47.65, 42.21, 38.84, 38.72, 37.17, 35.67, 30.40, 29.33, 28.27, 17.94.

HRMS (ESI): $C_{26}H_{38}N_6NaO_6^+$ *calcd*: 581.2807, *found*: 581.2808.

6.34 MC NP12/AA118 XZ34



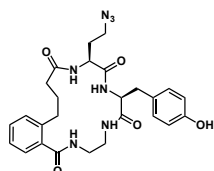
In a 500 mL flask **XZ28** (298.0 mg, 534 μ mol, 1.0 eq.) was dissolved in THF (250 mL) and DIPEA (326.0 μ L, 1.9 mmol, 3.5 eq.) was added, followed by HATU (406.0 mg, 1.1 mmol, 2.0 eq.). The mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed complete conversion to the desired product. The mixture was concentrated by rotary evaporation and purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 120 g, H $_2$ O:MeCN + 0.1% TFA, UV 200/215 nm) to yield the desired product as a white solid (148.0 mg, 51%). 32 mg of the product were purified again by preparative RP-HPLC (Method A) for final analytics and protein binding assays. Mixture of diastereomers with the ratio 50:38:12 (UPLC-MS analysis).

^1H NMR (500 MHz, DMSO- d_6) δ /ppm: 8.75 (dd, J = 7.0, 3.7 Hz, 1H), 8.39 (d, J = 7.1 Hz, 1H), 8.01 (d, J = 4.7 Hz, 1H), 7.85 (t, J = 5.6 Hz, 1H), 7.42 – 7.39 (m, 2H), 7.37 – 7.35 (m, 1H), 7.34 – 7.33 (m, 1H), 7.31 – 7.29 (m, 1H), 7.26 (dd, J = 5.3, 2.2 Hz, 2H), 7.21 (td, J = 7.4, 1.3 Hz, 1H), 5.35 (ddd, J = 10.2, 7.1, 2.9 Hz, 1H), 3.75 – 3.57 (m, 3H), 3.38 – 3.30 (m, 2H), 3.17 – 3.14 (m, 1H), 3.13 – 3.10 (m, 2H), 2.90 – 2.80 (m, 1H), 2.63 – 2.58 (m, 1H), 2.49 – 2.46 (m, 1H), 2.37 (dd, J = 15.5, 3.0 Hz, 1H), 1.72 – 1.62 (m, 1H), 1.64 – 1.53 (m, 1H), 1.13 (d, J = 6.7 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 175.93, 170.03, 169.33, 168.88, 140.21, 137.89, 137.63, 131.18, 129.42, 129.33, 129.16, 128.66, 127.72, 127.40, 125.93, 125.67, 51.25, 47.22, 40.98, 40.83, 39.17, 39.16, 38.99, 35.51, 29.13, 18.36.

HRMS (ESI): $\text{C}_{26}\text{H}_{30}\text{ClN}_7\text{NaO}_4^+$ *calcd*: 562.1940, *found*: 562.1940.

6.35 MC NP14/AA020 XZ35



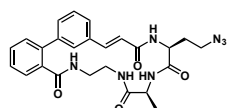
In a 1 L flask **XZ29** (967.0 mg, 1.8 mmol, 1.0 eq.) was dissolved in THF (800 mL) and DIPEA (1.1 mL, 6.3 mmol, 3.5 eq.) was added, followed by HATU (1.4 g, 3.6 mmol, 2.0 eq.). The mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed complete conversion to the desired product. The mixture was concentrated by rotary evaporation and purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, $\text{H}_2\text{O}:\text{MeCN} + 0.1\%$ TFA, UV 200/215 nm) to yield the desired product as a white solid (655.0 mg, **70%**). 38 mg of the product were purified again by preparative RP-HPLC (Method A) for final analytics and protein binding assays. Mixture of diastereomers with the ratio 43:57 (UPLC-MS analysis).

^1H NMR (500 MHz, DMSO- d_6) δ /ppm: 8.26 (d, J = 4.4 Hz, 1H), 7.99 (t, J = 5.6 Hz, 1H), 7.49 – 7.43 (m, 2H), 7.34 (td, J = 7.4, 1.7 Hz, 1H), 7.28 (dd, J = 7.5, 1.6 Hz, 1H), 7.25 (td, J = 7.3, 1.2 Hz, 2H), 6.96 – 6.92 (m, 2H), 6.64 – 6.61 (m, 2H), 4.32 – 4.29 (m, 1H), 3.78 (ddd, J = 8.1, 6.1, 4.3 Hz, 1H), 3.44 (ddt, J = 13.4, 5.4, 3.3 Hz, 1H), 3.37 – 3.33 (m, 2H), 3.18 – 3.07 (m, 3H), 3.02 – 2.95 (m, 1H), 2.85 (ddd, J = 13.9, 8.6, 5.5 Hz, 1H), 2.73 (dd, J = 14.4, 10.4 Hz, 1H), 2.64 (dt, J = 13.8, 8.1 Hz, 1H), 2.17 (t, J = 7.0 Hz, 2H), 1.90 – 1.82 (m, 1H), 1.74 – 1.63 (m, 2H), 1.64 – 1.56 (m, 1H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 174.56, 171.21, 171.01, 169.89, 155.79, 138.69, 137.68, 129.75, 129.68, 129.13, 128.19, 127.27, 125.84, 114.94, 53.93, 53.14, 47.08, 39.24, 38.82, 35.31, 33.29, 31.82, 29.75, 24.78.

HRMS (ESI): $\text{C}_{26}\text{H}_{31}\text{N}_7\text{NaO}_5^+$ *calcd*: 544.2279, *found*: 544.2279.

6.36 MC NP01/AA001 XZ36



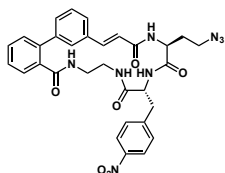
In a 1 L flask **XZ30** (764.0 mg, 1.5 mmol, 1.0 eq.) was dissolved in THF (700 mL) and DIPEA (787.0 μL , 4.5 mmol, 3.0 eq.) was added, followed by HATU (1.0 g, 2.7 mmol, 1.8 eq.). The mixture was stirred at RT for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The mixture was concentrated by rotary evaporation and purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, $\text{H}_2\text{O}:\text{MeCN} + 0.1\%$ TFA, UV 254/280 nm). Further purification was necessary due to co-eluted impurities. The impure material was purified by reversed phase preparative HPLC (Method A) followed by flash column chromatography on the ISOLERA (Silica, DCM:MeOH, UV). Product-containing fractions were combined and concentrated by rotary evaporation. The oily product was dissolved in MeCN/ H_2O and lyophilized to yield the desired product as a white solid (37.0 mg, **5%**). Mixture of diastereomers with the ratio 87:13 (UPLC-MS analysis).

^1H NMR (500 MHz, DMSO- d_6) δ /ppm: 8.47 (t, J = 5.4 Hz, 1H), 8.37 (d, J = 7.6 Hz, 1H), 7.74 – 7.66 (m, 2H), 7.61 (dt, J = 7.4, 1.7 Hz, 1H), 7.54 – 7.52 (m, 2H), 7.51 – 7.48 (m, 2H), 7.48 – 7.45 (m, 2H), 7.44 – 7.41 (m, 2H), 6.55 (d, J = 15.8 Hz, 1H), 4.22 (ddd, J = 13.8, 8.9, 6.7 Hz, 2H), 3.51 – 3.39 (m, 2H), 3.31 – 3.25 (m, 1H), 3.12 – 3.07 (m, 2H), 2.90 – 2.82 (m, 1H), 1.93 – 1.86 (m, 2H), 1.18 (d, J = 7.0 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 172.30, 170.85, 169.91, 166.04, 140.50, 140.02, 138.27, 137.81, 135.00, 129.49, 129.44, 129.30, 129.14, 129.05, 127.40, 127.12, 124.49, 123.14, 53.33, 48.61, 47.59, 39.60, 38.62, 29.99, 17.61.

HRMS (ESI): $\text{C}_{25}\text{H}_{27}\text{N}_7\text{NaO}_4^+$ *calcd*: 512.2017, *found*: 512.2011.

6.37 MC-NP01/AA058 XZ37



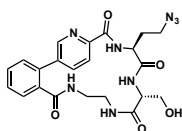
In a 50 mL flask **XZ31** (15.0 mg, 23.9 μmol , 1.0 eq.) was dissolved in DMF (20 mL) and DIPEA (12.5 μL , 71.6 μmol , 3.0 eq.) was added, followed by HATU (18.0 mg, 47.7 μmol , 2.0 eq.). The mixture was stirred at RT for 2 h after which UPLC-MS analysis showed full conversion of the starting material. The mixture was concentrated by rotary evaporation and purified by preparative reversed phase HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (13.0 mg, **89%**).

^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.61 – 8.59 (m, 1H), 8.57 (d, J = 8.5 Hz, 1H), 8.38 (d, J = 9.2 Hz, 1H), 8.13 – 8.12 (m, 2H), 7.61 – 7.58 (m, 1H), 7.57 – 7.54 (m, 2H), 7.53 – 7.52 (m, 2H), 7.48 – 7.47 (m, 4H), 7.43 – 7.41 (m, 2H), 7.41 – 7.39 (m, 1H), 6.47 (d, J = 15.9 Hz, 1H), 4.51 – 4.48 (m, 1H), 4.32 (q, J = 7.6 Hz, 1H), 3.28 – 3.26 (m, 1H), 3.19 – 3.17 (m, 2H), 3.17 – 3.14 (m, 2H), 3.13 – 3.11 (m, 1H), 2.95 (dd, J = 13.8, 10.2 Hz, 2H), 1.89 – 1.82 (m, 1H), 1.78 – 1.72 (m, 1H).

^{13}C NMR (600 MHz, 2D NMR, DMSO) δ /ppm: 170.52, 170.49, 169.97, 166.13, 146.76, 146.14, 140.0, 137.92, 137.82, 134.76, 130.30, 130.28, 129.25, 129.15, 128.90, 128.85, 128.54, 127.19, 126.84, 124.78, 122.89, 122.58, 53.53, 50.33, 47.32, 39.10, 38.11, 35.58, 28.75

HRMS (ESI): $\text{C}_{31}\text{H}_{30}\text{N}_8\text{NaO}_6^+$ *calcd*: 633.2181, *found*: 633.2176.

6.38 MC-NP07/AA026 XZ38



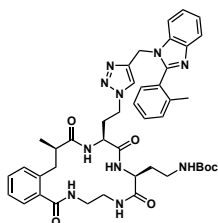
In a 500 mL flask **XZ32** (261.0 mg, 524.0 μmol , 1.0 eq.) was dissolved in THF (260 mL) and DIPEA (319.2 μL , 1.8 mmol, 3.5 eq.) was added, followed by HATU (398 mg, 1.1 mmol, 2.0 eq.). The mixture was stirred at RT for 1.5 h after which UPLC-MS analysis showed complete conversion to the desired product. The mixture was concentrated by rotary evaporation and purified by reversed phase flash column chromatography on the ISOLERA (RP-Silica, 340 g, $\text{H}_2\text{O}:\text{MeCN}$ +0.1% TFA, UV 254/280 nm) to yield the desired product as a white solid (79.0 mg, **31%**). 18 mg of the product were purified again by preparative RP-HPLC (Method A) for final analytics and protein binding assays. Mixture of diastereomers with the ratio 43:57 (UPLC-MS analysis).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 9.30 (d, J = 9.6 Hz, 1H), 8.56 (d, J = 2.6 Hz, 1H), 8.17 – 8.14 (m, 1H), 8.09 (dd, J = 8.0, 2.2 Hz, 1H), 7.67 (t, J = 4.9 Hz, 1H), 7.66 – 7.63 (m, 1H), 7.62 – 7.59 (m, 1H), 7.53 – 7.51 (m, 2H), 7.24 (d, J = 8.9 Hz, 1H), 6.92 (t, J = 5.3 Hz, 1H), 4.62 (td, J = 9.6, 5.1 Hz, 1H), 4.19 – 4.15 (m, 1H), 3.58 – 3.54 (m, 1H), 3.53 – 3.46 (m, 3H), 3.21 – 3.13 (m, 2H), 2.91 – 2.81 (m, 2H), 2.23 – 2.15 (m, 1H), 2.10 – 2.02 (m, 1H).

^{13}C NMR (500 MHz, 2D NMR, DMSO) δ /ppm: 170.6, 17.40, 169.24, 165.10, 147.99, 147.43, 137.54, 137.24, 134.38, 134.20, 129.64, 128.90, 128.30, 127.87, 121.79, 60.80, 54.84, 50.42, 47.73, 40.32, 39.07, 28.51.

HRMS (ESI): $\text{C}_{22}\text{H}_{24}\text{N}_8\text{NaO}_5^+$ *calcd*: 503.1762, *found*: 503.1764.

6.39 MC NP12/AA070/TA333 XZ39



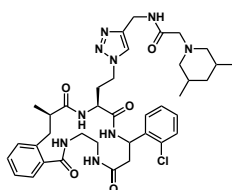
In a 10 mL flask **XZ33** (150.0 mg, 269.0 μmol , 1.0 eq.) and **TA333** (66.1 mg, 269.0 μmol , 1.0 eq.) were dissolved in DMSO (2.9 mL) and a solution of sodium ascorbate (31.9 mg, 161.0 μmol , 0.6 eq.) in H_2O (537 μL) was added. The solution was degassed by bubbling N_2 through the solution for 1 min. A solution of copper(II) sulfate pentahydrate (20.1 mg, 80.6 μmol , 0.3 eq.) in H_2O (403 μL) was added and the mixture was degassed again for 1 min. The reaction was stirred at RT for 2 h after which UPLC-MS analysis showed full conversion to the desired product. The solution was directly purified by preparative RP-HPLC (Method A, no TFA) and the product-containing fractions were lyophilized to yield the desired product as a white solid (142 mg, **66%**). Mixture of diastereomers in the ratio 87:6:7 (NMR).

^1H NMR (500 MHz, DMSO- d_6) δ /ppm: 8.24 (d, J = 5.3 Hz, 1H), 8.11 (t, J = 5.6 Hz, 1H), 7.91 (s, 1H), 7.72 – 7.67 (m, 2H), 7.65 (d, J = 8.1 Hz, 2H), 7.55 (dd, J = 7.6, 1.4 Hz, 1H), 7.48 (td, J = 7.5, 1.5 Hz, 1H), 7.41 (d, J = 7.6 Hz, 1H), 7.39 – 7.34 (m, 2H), 7.33 – 7.29 (m, 2H), 7.28 – 7.25 (m, 2H), 7.22 (td, J = 7.4, 1.2 Hz, 1H), 6.76 (t, J = 5.7 Hz, 1H), 5.33 (s, 2H), 4.36 (t, J = 7.2 Hz, 2H), 4.06 (ddd, J = 10.5, 8.0, 4.3 Hz, 1H), 3.61 (dt, J = 8.8, 5.8 Hz, 1H), 3.49 – 3.46 (m, 1H), 3.30 – 3.23 (m, 3H), 3.23 – 3.17 (m, 1H), 3.16 – 3.10 (m, 1H), 2.91 (ddt, J = 20.5, 13.6, 5.9 Hz, 2H), 2.68 – 2.57 (m, 1H), 2.27 – 2.16 (m, 2H), 2.15 (s, 3H), 2.04 – 1.96 (m, 1H), 1.75 – 1.66 (m, 1H), 1.31 (s, 9H), 1.14 (d, J = 7.0 Hz, 3H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 176.72, 171.08, 171.02, 169.54, 155.54, 152.28, 141.80, 140.64, 138.00, 137.69, 137.29, 134.10, 130.50, 130.22, 129.51, 129.09, 128.77, 128.48, 127.48, 125.86, 125.80, 123.63, 122.90, 122.60, 118.48, 111.47, 77.52, 53.40, 51.12, 46.60, 42.25, 39.40, 38.78, 38.77, 37.10, 35.68, 30.72, 30.47, 28.24, 19.43, 17.83.

HRMS (ESI): $\text{C}_{43}\text{H}_{53}\text{N}_{10}\text{O}_6^+$ *calcd*: 805.4144, *found*: 805.4142.

6.40 MC NP12/AA118/TA622 XZ40



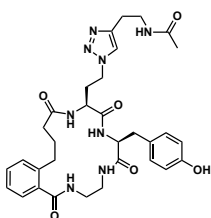
In a 10 mL flask **XZ34** (100.0 mg, 185.0 μmol , 1.0 eq.) and **TA622** (38.6 mg, 185.0 μmol , 1.0 eq.) were dissolved in DMSO (2 mL) and a solution of sodium ascorbate (22.0 mg, 111.0 μmol , 0.6 eq.) in H_2O (370 μL) was added. The solution was degassed by bubbling N_2 through the solution for 1 min. A solution of copper(II) sulfate pentahydrate (13.9 mg, 55.6 μmol , 0.3 eq.) in H_2O (278 μL) was added and the mixture was degassed again for 1 min. The reaction was stirred at RT for 14 h after which UPLC-MS analysis showed full conversion to the desired product. The solution was directly purified by preparative RP-HPLC (Method A) and the product-containing fractions were lyophilized to yield the desired product as a white solid (129 mg, **93%**). Mixture of diastereomers in the ratio 57:31:11 (UPLC). NMR showed the protonated species of the tertiary amine sidechain.

^1H NMR (500 MHz, DMSO- d_6) δ /ppm: 9.77 (s, 1H), 9.09 – 9.00 (m, 1H), 8.81 (dd, J = 6.8, 3.8 Hz, 1H), 8.31 (d, J = 7.3 Hz, 1H), 8.14 (d, J = 5.7 Hz, 1H), 7.93 (t, J = 5.7 Hz, 1H), 7.87 (s, 1H), 7.47 – 7.42 (m, 1H), 7.42 – 7.40 (m, 1H), 7.40 – 7.38 (m, 1H), 7.38 – 7.35 (m, 1H), 7.32 – 7.23 (m, 4H), 5.40 (ddd, J = 10.3, 7.3, 3.2 Hz, 1H), 4.37 (t, J = 5.7 Hz, 2H), 4.18 (ddd, J = 14.2, 9.0, 5.6 Hz, 1H), 4.10 (ddd, J = 13.8, 8.9, 6.6 Hz, 1H), 3.87 (s, 2H), 3.80 – 3.78 (m, 1H), 3.69 – 3.65 (m, 1H), 3.52 – 3.43 (m, 1H), 3.34 – 3.30 (m, 2H), 3.29 – 3.24 (m, 1H), 3.24 – 3.20 (m, 1H), 3.19 – 3.11 (m, 1H), 2.92 – 2.82 (m, 1H), 2.64 (dd, J = 13.6, 6.5 Hz, 1H), 2.59 – 2.53 (m, 2H), 2.49 – 2.35 (m, 2H), 2.12 – 2.03 (m, 1H), 2.00 – 1.93 (m, 2H), 1.93 – 1.88 (m, 1H), 1.73 (d, J = 13.0 Hz, 1H), 1.15 (d, J = 6.8 Hz, 3H), 0.86 (d, J = 6.6 Hz, 6H), 0.76 (q, J = 12.2 Hz, 1H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 175.89, 169.77, 169.38, 168.95, 163.93, 143.62, 140.17, 137.85, 136.99, 131.19, 129.44, 129.40, 129.37, 128.65, 127.92, 127.64, 127.39, 125.80, 122.98, 57.75, 56.79, 51.03, 47.46, 46.23, 40.87, 40.81, 39.39, 39.18, 38.55, 35.78, 34.23, 30.73, 28.15, 18.26, 17.97.

HRMS (ESI): $\text{C}_{38}\text{H}_{51}\text{ClN}_9\text{O}_5^+$ *calcd*: 748.3696, *found*: 748.3685.

6.41 MC NP14/AA020/TA607 XZ41



In a 10 mL flask **XZ35** (150.0 mg, 288.0 μmol , 1.0 eq.) and **TA607** (32.0 mg, 288.0 μmol , 1.0 eq.) were dissolved in DMSO (3 mL) and a solution of sodium ascorbate (34.2 mg, 173.0 μmol , 0.6 eq.) in H_2O (575 μL) was added. The solution was degassed by bubbling N_2 through the solution for 1 min. A solution of copper(II) sulfate pentahydrate (21.5 mg, 86.3 μmol , 0.3 eq.) in H_2O (431 μL) was added and the mixture was degassed again for 1 min. The reaction was stirred at RT for 2 h after which UPLC-MS analysis showed full conversion to the desired product. The solution was directly purified by preparative RP-HPLC (Method A) and the product-containing fractions were lyophilized to yield the desired product as a white solid (90 mg, **50%**). Mixture of diastereomers in the ratio 27:23:50 (UPLC).

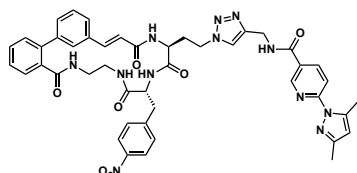
^1H NMR (500 MHz, DMSO- d_6) δ /ppm: 8.37 (d, J = 4.6 Hz, 1H), 8.00 (t, J = 5.6 Hz, 1H), 7.93 (t, J = 5.7 Hz, 1H), 7.74 (s, 1H), 7.54 (d, J = 8.4 Hz, 1H), 7.47 (t, J = 5.6 Hz, 1H), 7.34 (td, J = 7.4, 1.8 Hz, 1H), 7.26 (dtd, J = 18.1, 7.5, 1.5 Hz, 3H), 6.97 – 6.92 (m, 2H), 6.64

– 6.60 (m, 2H), 4.30 (ddd, $J = 10.2, 8.4, 4.5$ Hz, 1H), 4.25 – 4.19 (m, 1H), 4.15 – 4.11 (m, 1H), 3.67 (ddd, $J = 8.2, 6.2, 4.5$ Hz, 1H), 3.44 (dtd, $J = 13.5, 5.6, 5.2, 2.7$ Hz, 1H), 3.38 – 3.33 (m, 2H), 3.30 – 3.25 (m, 2H), 3.11 (dd, $J = 14.4, 4.4$ Hz, 1H), 3.04 – 2.96 (m, 1H), 2.84 (ddd, $J = 14.1, 8.7, 5.5$ Hz, 1H), 2.78 – 2.72 (m, 3H), 2.65 (dt, $J = 13.8, 7.8$ Hz, 1H), 2.19 (t, $J = 6.9$ Hz, 2H), 2.04 – 1.94 (m, 2H), 1.86 (ddd, $J = 13.7, 8.5, 7.1$ Hz, 1H), 1.79 (s, 3H), 1.74 – 1.65 (m, 1H).

^{13}C NMR (101 MHz, DMSO) δ /ppm: 174.60, 171.15, 170.99, 169.88, 169.25, 155.73, 144.34, 138.71, 137.67, 129.72, 129.46, 129.14, 128.22, 127.26, 125.83, 122.43, 115.01, 54.07, 52.98, 45.97, 39.06, 38.78, 38.42, 35.26, 33.35, 31.83, 31.08, 25.58, 24.90, 22.64.

HRMS (ESI): $\text{C}_{32}\text{H}_{40}\text{N}_8\text{NaO}_6^+$ *calcd*: 655.2963, *found*: 655.2964.

6.42 MC-NP01/AA058/TA229 XZ42



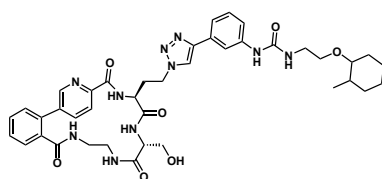
In a 1.5 mL Eppendorf tube **XZ37** (10.0 mg, 16.4 μmol , 1.0 eq.) and **TA229** (4.2 mg, 16.4 μmol , 1.0 eq.) were dissolved in DMSO (200 μL) and a solution of sodium ascorbate (2.0 mg, 9.8 μmol , 0.6 eq.) in H_2O (32.8 μL) was added. The solution was degassed by bubbling N_2 through the solution for 1 min. A solution of copper(II) sulfate pentahydrate (1.2 mg, 4.9 μmol , 0.3 eq.) in H_2O (24.6 μL) was added and the mixture was degassed again for 1 min. The reaction was agitated at RT for 12 h after which UPLC-MS analysis showed full conversion to the desired product. The solution was directly purified by preparative RP-HPLC (Method A) and the product-containing fractions were lyophilized to yield the desired product as a white solid (5 mg, **35%**). Mixture of diastereomers in the ratio 61:39 (UPLC)/47:42:6:5 (NMR).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.24 – 9.19 (m, 1H), 8.89 (d, $J = 3.3$ Hz, 1H), 8.64 (d, $J = 8.4$ Hz, 1H), 8.61 – 8.55 (m, 1H), 8.34 (dt, $J = 7.0, 4.3$ Hz, 1H), 8.27 (d, $J = 9.2$ Hz, 1H), 8.11 (t, $J = 8.3$ Hz, 2H), 8.00 (s, 1H), 7.91 – 7.89 (m, 1H), 7.65 (t, $J = 5.6$ Hz, 1H), 7.63 – 7.59 (m, 1H), 7.55 – 7.54 (m, 1H), 7.53 – 7.50 (m, 2H), 7.49 – 7.47 (m, 2H), 7.47 – 7.45 (m, 2H), 7.43 – 7.41 (m, 2H), 7.41 – 7.37 (m, 2H), 6.55 (d, $J = 15.8$ Hz, 1H), 6.16 (s, 1H), 4.56 – 4.53 (m, 2H), 4.52 – 4.47 (m, 1H), 4.28 – 4.23 (m, 1H), 4.23 – 4.18 (m, 2H), 3.20 – 3.17 (m, 2H), 3.13 – 3.08 (m, 2H), 2.96 – 2.87 (m, 2H), 2.61 (s, 3H), 2.21 (s, 3H), 2.10 – 2.02 (m, 1H), 2.00 – 1.92 (m, 1H).

^{13}C NMR (500 MHz, 2D NMR, DMSO) δ /ppm: 175.58, 169.91, 165.81, 165.69, 163.64, 154.18, 149.45, 146.76, 146.08, 145.84, 145.80, 144.42, 141.08, 139.93, 137.76, 137.62, 134.50, 130.17, 130.14, 129.53, 128.95, 128.47, 127.13, 126.85, 126.16, 124.76, 123.84, 122.84, 122.74, 122.59, 122.45, 113.83, 109.57, 53.29, 50.13, 45.89, 39.11, 36.52, 35.71, 34.54, 30.68, 14.30, 13.06.

HRMS (ESI): $\text{C}_{45}\text{H}_{44}\text{N}_{12}\text{NaO}_7^+$ *calcd*: 887.3348, *found*: 887.3346.

6.43 MC-NP07/AA026/TA256 XZ43



In a 2.0 mL Eppendorf tube **XZ38** (45.0 mg, 93.7 μmol , 1.0 eq.) and **TA256** (28.1 mg, 93.7 μmol , 1.0 eq.) were dissolved in DMSO (990 μL) and a solution of sodium ascorbate (11.1 mg, 56.2 μmol , 0.6 eq.) in H_2O (187 μL) was added. The solution was degassed by bubbling N_2 through the solution for 1 min. A solution of copper(II) sulfate pentahydrate (7.0 mg, 28.1 μmol , 0.3 eq.) in H_2O (140 μL) was added and the mixture was degassed again for 1 min. The reaction was agitated at RT for 2 h after which UPLC-MS analysis showed full conversion to the desired product. The solution was directly purified by preparative RP-HPLC (Method A) and the product-containing fractions were lyophilized to yield the desired product as a white solid (60 mg, **82%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 9.40 (d, $J = 9.5$ Hz, 1H), 8.73 (s, 1H), 8.57 (d, $J = 8.5$ Hz, 1H), 8.47 (t, $J = 5.5$ Hz, 1H), 8.17 (d, $J = 8.0$ Hz, 1H), 8.09 (dd, $J = 8.0, 2.2$ Hz, 1H), 7.94 – 7.88 (m, 1H), 7.70 – 7.65 (m, 1H), 7.64 (s, 1H), 7.63 – 7.54 (m, 1H), 7.53 (d, $J = 4.2$ Hz, 1H), 7.51 – 7.45 (m, 1H), 7.39 – 7.31 (m, 2H), 7.31 – 7.28 (m, 1H), 7.28 – 7.21 (m, 1H), 6.92 (t, $J = 5.4$ Hz, 1H), 6.13 (t, $J = 6.0$ Hz, 1H), 4.58 – 4.48 (m, 2H), 4.21 – 4.10 (m, 1H), 3.59 – 3.53 (m, 2H), 3.46 (dd, $J = 10.8, 4.9$ Hz, 1H), 3.42 – 3.29 (m, 2H), 3.28 – 3.05 (m, 4H), 2.91 – 2.81 (m, 1H), 2.81 – 7.23 (m, 1H), 2.66 – 2.54 (m, 1H), 2.49 – 2.37 (m, 1H), 2.34 – 2.15 (m, 1H), 2.01 (dt, $J = 12.6, 4.2$ Hz, 1H), 1.73 – 1.67 (m, 1H), 1.67 – 1.61 (m, 1H), 1.58 – 1.49 (m, 1H), 1.40 – 1.29 (m, 1H), 1.24 – 1.11 (m, 2H), 1.11 – 1.02 (m, 1H), 0.96 (d, $J = 6.5$ Hz, 3H).

^{13}C NMR (500 MHz, 2D NMR, DMSO) δ /ppm: 172.64, 170.34, 169.19, 165.29, 154.92, 150.59, 148.03, 147.49, 146.19, 140.86, 137.55, 137.27, 136.26, 134.28, 131.03, 129.65, 129.03, 128.91, 128.32, 121.82, 121.43, 117.91, 116.83, 113.99, 82.89, 67.07, 60.77, 54.87, 50.39, 46.56, 40.37, 39.40, 39.09, 37.57, 33.05, 30.66, 29.84, 24.86, 24.22, 18.49.

HRMS (ESI): $\text{C}_{40}\text{H}_{48}\text{N}_{10}\text{NaO}_7^+$ *calcd*: 803.3600, *found*: 803.3585.

7. Macrocycle Binding Evaluation

7.1 Differential Scanning Fluorimetry (DSF)

Stock solutions:

MC NP12/AA118 **XZ34** 500 μM (PBS buffer)
 MC NP14/AA020 **XZ35** 500 μM (PBS buffer)
 MC NP07/AA026 **XZ38** 500 μM (PBS buffer)
 MC NP12/AA118/TA622 **XZ40** 500 μM (PBS buffer)
 MC NP14/AA020/TA607 **XZ41** 500 μM (PBS buffer)
 AGP 10 mg/ml (PBS buffer)

MC NP12/AA070 **XZ33** 500 μM (5% DMSO/PBS buffer)
 MC NP01/AA001 **XZ36** 250 μM (25% DMSO/PBS buffer)
 MC NP07/AA026/TA256 **XZ43** 500 μM (25% DMSO/PBS buffer)
 MC NP01/AA058 **XZ37** 250 μM (25% DMSO/PBS buffer)
 MC NP01/AA058/TA229 **XZ42** 250 μM (25% DMSO/PBS buffer)
 HSA 10 mg/ml (PBS buffer)

Sample preparation:

The MC stock solution (97.5 μL) was mixed with the protein solution (2.5 μL), giving a final protein concentration of 0.25 mg/ml and 487.5 μM MC concentration in the according buffer. For every buffer system, a reference sample (buffer + protein) was prepared. All MCs were tested against HSA and AGP (**XZ37** and **XZ42** were not completely soluble). The measurements were performed in triplicates on a Prometheus NT.48 with a rate of 1.5 $^{\circ}\text{C}$ per minute in a temperature range from 20 $^{\circ}\text{C}$ - 95 $^{\circ}\text{C}$.

Remark: **XZ39** could not be tested due to high fluorescence activity of the compound. AGP (10 mg/ml, 1 mL) was dialyzed against PBS (3 x 1000 mL, 4 $^{\circ}\text{C}$, 3 x 18 h) prior to the binding assay.

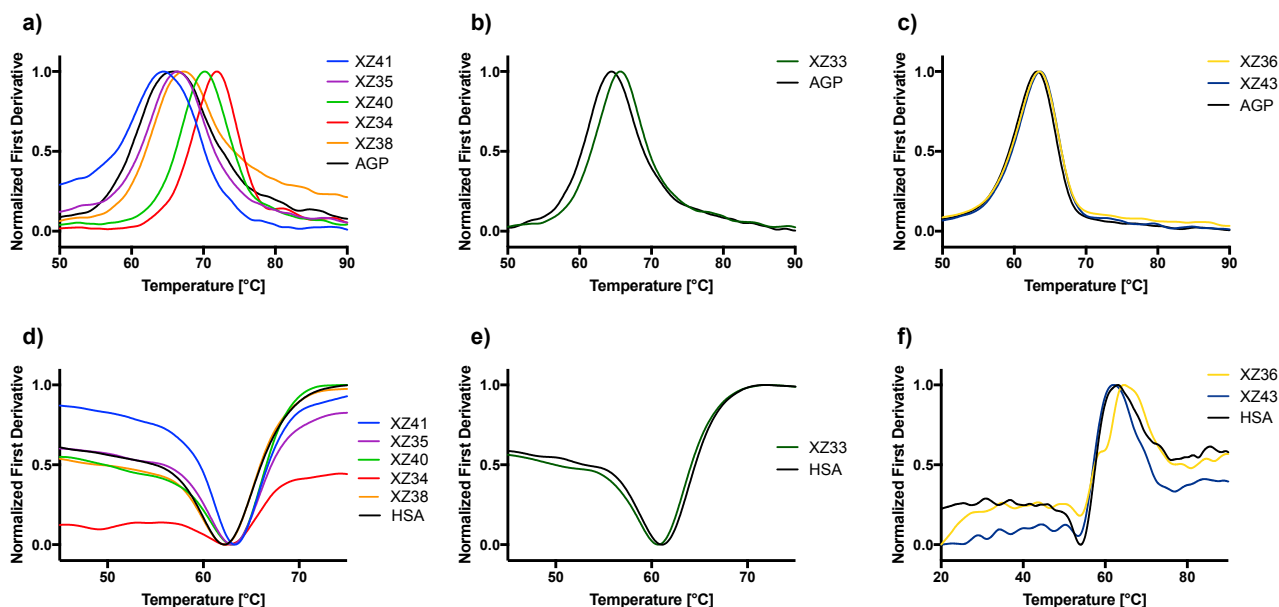


Figure 24. Normalized first derivatives of the DSF measurements. **a)** Measurements of AGP with MC ligands in PBS buffer. **b)** Measurement of AGP with MC ligand **XZ33** in 5% DMSO in PBS. **c)** Measurements of AGP with MC ligands in 25% DMSO in PBS. **d)** Measurements of HSA with MC ligands in PBS buffer. **e)** Measurement of HSA with MC ligand **XZ33** in 5% DMSO in PBS. **f)** Measurements of AGP with MC ligands in 25% DMSO in PBS.

Table 8. Found ΔT_m shifts during macrocycle-protein binding events. Positive ΔT_m values imply stabilization of the protein structure, negative ΔT_m values imply a destabilization of the protein structure. Macrocycles **XZ37** and **XZ42** are not shown in this analysis due to their poor solubility, which resulted in 0 $^{\circ}\text{C}$ shift of the protein melting temperature.

Compound	ΔT_m for AGP [$^{\circ}\text{C}$]	ΔT_m for HSA [$^{\circ}\text{C}$]	% DMSO	Comments
XZ33	1.16	-0.44	5	

XZ34	5.94	N/A ^[a]	0	AGP Binder
XZ40	4.32	0.67	0	AGP Binder
XZ35	0.49	0.83	0	
XZ35^[b]	0.37	-	5	
XZ41	-1.21	1.27	0	
XZ41^[b]	-0.78	-	5	
XZ36	0.32	9.47 ^[c]	25	Bad peak shape
XZ38	1.37	-0.07	0	
XZ38^[b]	1.38	-	5	
XZ43	0.32	8.65 ^[c]	25	Bad peak shape

[a] Peak is too broad to determine a ΔT_m value. [b] Due to a broadened peak shape of pure AGP in PBS the assay was repeated in 5% DMSO in PBS which showed a sharper melting peak. [c] Unreliable data. Curve shape is too unsteady for accurate melting temperature shift measurements.

From the found temperature shifts we chose **XZ34**, **XZ39** (no DSF measurements possible), **XZ40** and **XZ41** for ITC measurements with AGP and **XZ36**, **XZ40** and **XZ41** for ITC measurements with HSA.

7.2 Isothermal Calorimetry (ITC)

General:

For ITC measurements the protein solution (50 μ M in PBS, approx. 300 μ L) was placed in the sample cell and the ligand (macrocycle) solution (500 μ M in PBS, approx. 40 μ L) was placed in the syringe. The titrations were performed at 25°C or 10°C with a stirring rate of 750 rpm. Reference power was set to 6 μ cal/s. In total 17 injections were performed with an initial delay of 300 s. The added volume per injection was 2.3 μ L over a duration of 4.6 s (first injection 0.5 μ L in 1.0 s) with a spacing of 150 s between the additions and a filter time of 2 s. The data was analyzed by NitPick and Sedphat for fitting and K_D determinations.

XZ34 versus AGP:

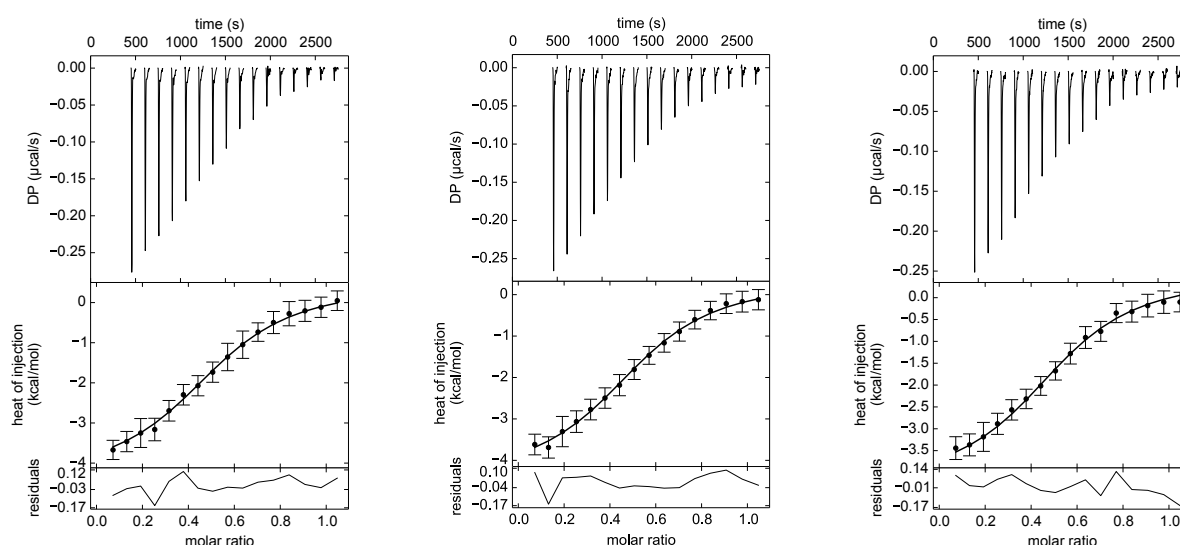


Figure 25. The three ITC titrations of **XZ34** versus AGP. All graphs were analyzed and integrated using NITPIC. For the physicochemical calculations all three measurements were fitted and processed using SEDPHAT.

For optimal binding constant analysis ligand **XZ34** was used as a 260 μM solution in PBS buffer with 50 μM AGP in PBS buffer at 25°C. Triplicate measurements were conducted as well as the reference titration (**XZ34** into PBS buffer). After subtraction of the reference titration from the binding measurements, the data was globally fitted to give the following calculated values:

$K_D = 4.1 \mu\text{M}$ with a confidence interval (95%) from 2.9 - 6.1 μM .

$\Delta H = -4.78 \text{ kcal/mol} = -20.01 \text{ kJ/mol}$ with a confidence interval (95%) from -5.51 - -4.28 kcal/mol.

$\Delta G = -30.75 \text{ kJ/mol}$ from $\Delta G = RT \ln(K_D) = \Delta H - T\Delta S$ with $R = 8.314472 \text{ J mol}^{-1} \text{ K}^{-1}$ and $T = 298.15 \text{ K}$.

$\Delta S = 36.01 \text{ J mol}^{-1} \text{ K}^{-1}$

XZ39 and XZ41 versus AGP:

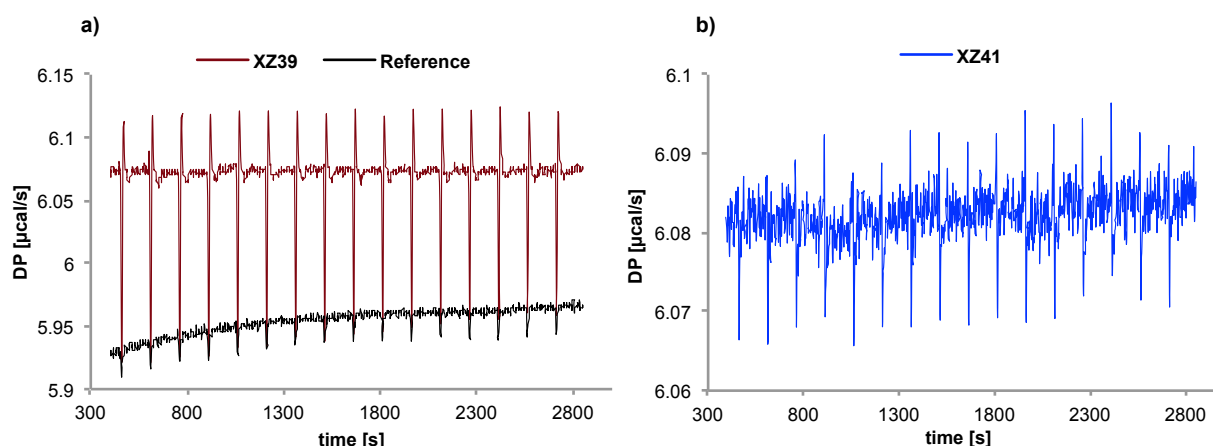


Figure 26. a) ITC titration of **XZ39** versus AGP with the reference titration **XZ39** vs PBS buffer. The data was not analyzed and integrated. b) ITC titration of **XZ41** versus AGP. The data was not analyzed and integrated.

The titrations were performed at 50 μM AGP and 260 μM macrocycle ligand (5% DMSO in PBS for **XZ39**, pure PBS for **XZ41**) in single measurements at 25°C. A reference titration of **XZ39** was conducted to evaluate the solvation enthalpy. The measurements were not further improved nor the data analyzed due to the very weak binding. We assumed no binding for **XZ41** due to the very weak differential power (DP) changes, that probably uniquely arose from the solvation enthalpy

XZ40 versus AGP:

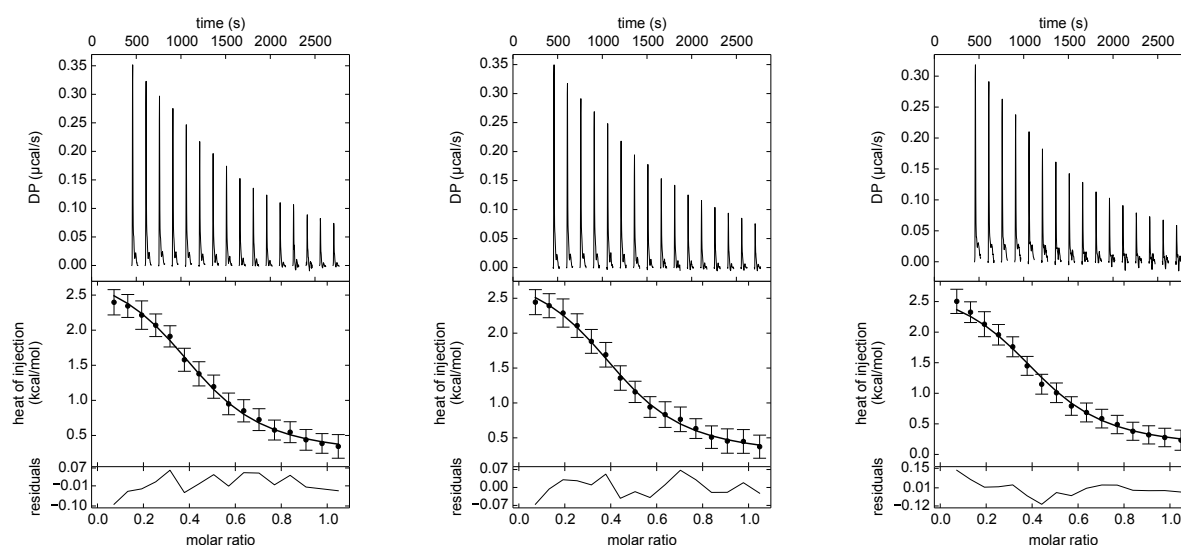


Figure 27. The three ITC titrations of **XZ40** versus AGP. All graphs were analyzed and integrated using NITPIC. For the physicochemical calculations all three measurements were fitted and processed using SEDPHAT.

The optimal conditions were found to be 100 μM AGP and 520 μM **XZ40** in PBS buffer. Triplicate measurements were performed with a reference titration. Due to the small binding enthalpies the temperature of the assay was lowered to 10°C. After subtraction of the reference titration from the binding measurements, the data was globally fitted to give the following calculated values:

$K_D = 7.0 \mu\text{M}$ with a confidence interval (95%) from 4.7 - 10.7 μM .

$\Delta H = 2.72 \text{ kcal/mol} = 11.39 \text{ kJ/mol}$ with a confidence interval (95%) from 2.43 - 3.14 kcal/mol.

$\Delta G = -29.96 \text{ kJ/mol}$ from $\Delta G = RT \ln(K_D) = \Delta H - T\Delta S$ with $R = 8.314472 \text{ J mol}^{-1} \text{ K}^{-1}$ and $T = 283.15 \text{ K}$.

$\Delta S = 138.97 \text{ J mol}^{-1} \text{ K}^{-1}$

XZ36, **XZ40** and **XZ41** versus HSA:

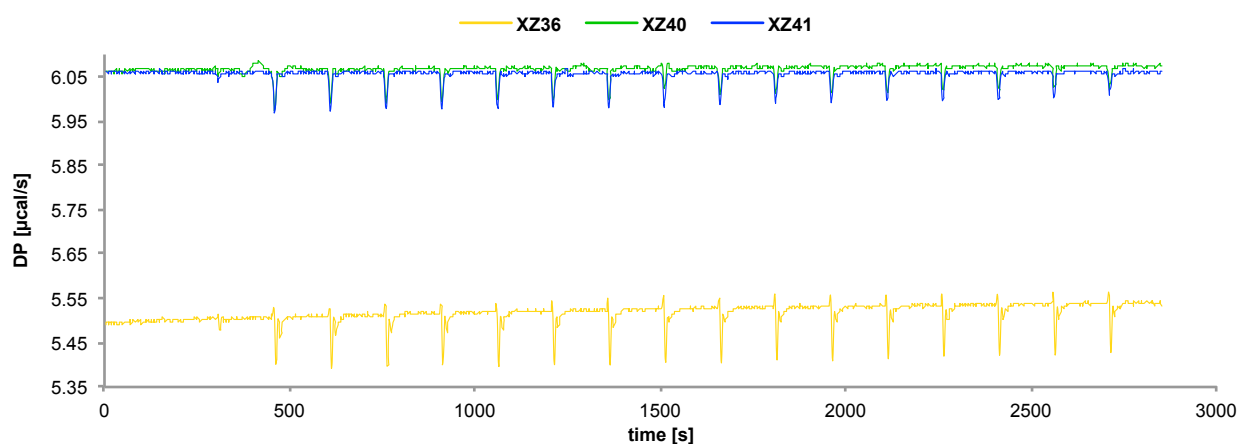


Figure 28. ITC titrations of **XZ36**, **XZ40** and **XZ41** versus HSA. The data was not analyzed and integrated. Reference titrations not shown.

The **XZ36** versus HSA assay was performed at 25 μM HSA in 25% DMSO/PBS and 250 μM macrocycle ligand in 25% DMSO/PBS at 25°C. Care was taken to exactly match the DMSO/PBS ratios in all samples and buffers.

XZ40 and **XZ41** versus HSA measurements were performed at 50 μM /500 μM in PBS buffer at 25°C.

In all three assays no strong binding events were observed. Therefore, no further measurement optimizations and analyses were conducted.

8. Quantitative PCR (qPCR) of Chemical Reactions on DNA

General Procedure

DNA damage evaluations were made with representative DNA strands, based on the same structure as the coding DNA strands of the DEML. For the evaluation of chemical modifications on a single-stranded DNA we used DNA with the sequence 5'-GGAGCTTGTGAATTCTGGATGGGACGTGTGTGAATTGTCTTTTGTGTGCGGATCCAAGTTCGGTGAATGGATTTTTTACTACGGA TGGATACTCT-3' (**DNA1**).

Chemical modifications on a double stranded system were evaluated on a DNA pair consisting of **DNA1** annealed to its reverse complementary strand with the sequence 5'-AGAGTATCCATCCGTAGTAAAAATCCATTCACCGAAGTTCGGATCCGCA CACAAAAGACAATTCACACACGTCCCATCCAGAATTCACAAGCTCC-3' (**DNA2**).

The annealing of **DNA1** and **DNA2** was performed with the following gradient: 95°C(1 min)-70°C(2 min)-45°C(2 min) to yield the double-stranded **DNA3**. **DNA1** and **DNA3** were diluted with H₂O to a final concentration of 0.45 ng/ μL . **Primer1** had the following sequence: 5'-AGAGTATCCATCCGTAGT-3' and **Primer2**: 5'-GGAGCTTGTGAATTCTGG-3'.

For the qPCR assay the following solutions were mixed in a 96-well plate: **DNA1** or **DNA3** (3 μL , 0.45 ng/ μL in H₂O), **Primer1** (0.5 μL , 2.5 μM in H₂O), **Primer2** (0.5 μL , 2.5 μM in H₂O), H₂O (3.5 μL) and SYBR Green Master Mix (5 μL). After mixing and centrifugation of the plate, the PCR was run according to the following program: 95°C(2 min)-40 x (95°C(15 s)-60°C(1 min)). The progress of the DNA amplification was observed by fluorescence emission of the DNA-SYBR complex formed during the PCR. All assays were performed with three or more valid replicates that were further processed. Standard curves with unmodified **DNA1** or **DNA3** at four concentrations (1x, 10x, 100x, 1000x dilutions) were generated as well as dummy samples (no primers and primer only).

The quantity of remaining intact DNA could be calculated by the number of PCR cycles necessary for complete consumption of the primers of each assay relative to the generated standard curves. For this purpose the standard curves were fitted with logarithmic trendlines in Excel. For single stranded assays we calculated the following trendline: $y = -4.384 \ln(x) + 7.4961$, $R^2 = 0.99126$, with y = the number of PCR cycles and x = the remaining intact DNA quantity. For double-stranded DNA we calculated the trendline: $y = -3.421 \ln(x) + 11.458$, $R^2 = 0.98647$.

Ester Deprotection Conditions

In a PCR tube **DNA1** (3.0 μ L, 110 μ M in H₂O, 1.0 eq.) was mixed with H₂O (2 μ L), MeCN (3.5 μ L) and a solution of LiOH hydrate (1.65 μ L, 100 mM in H₂O, 500 eq.). The mixture was left standing at RT for 16 h, followed by EtOH purification. The washed DNA pellet was dissolved in H₂O (30 μ L) and further diluted to the desired concentration for qPCR.

Click Reaction Conditions

In a PCR tube **DNA1** (3.0 μ L, 110 μ M in H₂O, 1.0 eq.) was mixed with TEAA (1 M, pH 7.2, 3 μ L), DMSO (7 μ L), a solution of TA662 (3.3 μ L, 10 mM in DMSO, 100 eq.) and a solution of sodium ascorbate (3.3 μ L, 20 mM in H₂O, 200 eq.). The mixture was degassed for 30 s by bubbling a stream of N₂ gas through the solution. Cu(II)-TBTA complex solution (6.6 μ L, 10 mM in 55% DMSO, 200 eq.) was added and the mixture was degassed again for 30 s. The mixture was left standing at RT for 16 h, followed by EtOH purification. The washed DNA pellet was dissolved in H₂O (30 μ L) and further diluted to the desired concentration for qPCR. For the double-stranded assay **DNA1** was replaced by **DNA3** (6.0 μ L, 50 μ M, 1.0 eq.)

DMTMM Coupling with Excess Amine Conditions

In a PCR tube **DNA1** (3.0 μ L, 110 μ M in H₂O, 1.0 eq.) was mixed with MOPS buffer (300 mM, pH 8.2, 0.5 μ L), NMM (2.2 μ L, 300 mM in DMSO, 2000 eq.), AA001 (3.3 μ L, 100 mM in DMSO, 1000 eq.) and DMTMM-BF₄ (1.7 μ L, 200 mM in DMSO, 1000 eq.). The mixture was left standing at RT for 16 h, followed by EtOH purification. The washed DNA pellet was dissolved in H₂O (30 μ L) and further diluted to the desired concentration for qPCR. For the double-stranded assay **DNA1** was replaced by **DNA3** (6.0 μ L, 50 μ M, 1.0 eq.)

DMTMM Coupling with Excess Acid Conditions

In a PCR tube **DNA1** (3.0 μ L, 110 μ M in H₂O, 1.0 eq.) was mixed with MOPS buffer (300 mM, pH 8.2, 0.5 μ L), NMM (2.2 μ L, 300 mM in DMSO, 2000 eq.), 1-*H*-indazole-3-carboxylic acid (3.3 μ L, 100 mM in DMSO, 1000 eq.) and DMTMM-BF₄ (1.7 μ L, 200 mM in DMSO, 1000 eq.). The mixture was left standing at RT for 16 h, followed by EtOH purification. The washed DNA pellet was dissolved in H₂O (30 μ L) and further diluted to the desired concentration for qPCR. For the double-stranded assay **DNA1** was replaced by **DNA3** (6.0 μ L, 50 μ M, 1.0 eq.)

EDC/HOAt Coupling with Excess Amine Conditions

In a PCR tube **DNA1** (3.0 μ L, 110 μ M in H₂O, 1.0 eq.) was mixed with MOPS buffer (300 mM, pH 8.2, 0.5 μ L), DIPEA (3.7 μ L, 180 mM in DMSO, 2000 eq.), AA001 (3.3 μ L, 100 mM in DMSO, 1000 eq.), EDC hydrochloride (1.7 μ L, 200 mM in DMSO, 1000 eq.) and HOAt (1.7 μ L, 200 mM in DMSO, 1000 eq.). The mixture was left standing at RT for 16 h, followed by EtOH purification. The washed DNA pellet was dissolved in H₂O (30 μ L) and further diluted to the desired concentration for qPCR. For the double-stranded assay **DNA1** was replaced by **DNA3** (6.0 μ L, 50 μ M, 1.0 eq.)

EDC/HOAt Coupling with Excess Acid Conditions

In a PCR tube **DNA1** (3.0 μ L, 110 μ M in H₂O, 1.0 eq.) was mixed with MOPS buffer (300 mM, pH 8.2, 0.5 μ L), DIPEA (3.7 μ L, 180 mM in DMSO, 2000 eq.), 1-*H*-indazole-3-carboxylic acid (3.3 μ L, 100 mM in DMSO, 1000 eq.), EDC hydrochloride (1.7 μ L, 200 mM in DMSO, 1000 eq.) and HOAt (1.7 μ L, 200 mM in DMSO, 1000 eq.). The mixture was left standing at RT for 16 h, followed by EtOH purification. The washed DNA pellet was dissolved in H₂O (30 μ L) and further diluted to the desired concentration for qPCR. For the double-stranded assay **DNA1** was replaced by **DNA3** (6.0 μ L, 50 μ M, 1.0 eq.)

NOSYL Deprotection Conditions

In a PCR tube **DNA1** (3.0 μ L, 110 μ M in H₂O, 1.0 eq.) was mixed with MOPS buffer (600 mM, pH 8.2, 3.0 μ L), DBU (6.0 μ L, 300 mM in DMSO, 5455 eq.) and BME (6.0 μ L, 300 mM in DMSO, 5455 eq.). The mixture was degassed by bubbling N₂ through the solution for 30 s and then left standing at RT for 16 h, followed by EtOH purification. The washed DNA pellet was dissolved in H₂O (30 μ L) and further diluted to the desired concentration for qPCR.

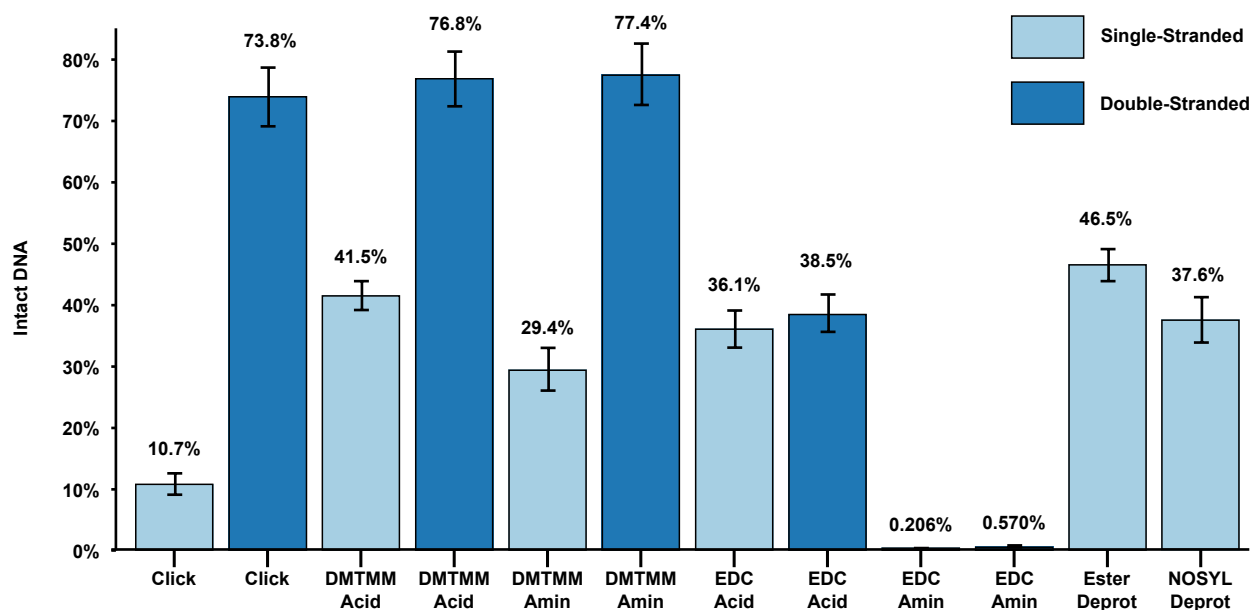
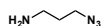


Figure 29. qPCR results from all tested reaction conditions with single- or double-stranded DNA. Not all of these conditions were applied during DEML assembly, but some are routinely used in DECL synthesis (e.g. EDC acid coupling).

9. Synthesis and Codon Sequences of the Building Blocks

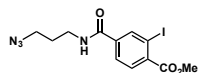
9.1 Synthesis of 3-azidopropan-1-amine XZ44



3-Bromopropan-1-amine hydrobromide (5.0 g, 22.8 mmol, 1.0 eq.) and sodium azide (4.5 g, 68.5 mmol, 3.0 eq.) were dissolved in H₂O (20 mL) and heated to 80°C for 22 h. After cooling a solution of KOH (6 g, 106.9 mmol, 4.7 eq.) in H₂O (10 mL) was added while cooling in an ice bath. The solution was extracted with DCM (4 x 50 mL) and the combined organic layers were washed with brine (50 mL) and dried over Na₂SO₄. The solvent was removed *in vacuo* (40°C, 250 mbar) to yield the desired product as slightly yellow liquid (2.0 g, **87.4%**). Analytical data was in agreement with reported data.^[13]

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 3.42 (t, *J* = 6.6 Hz, 2H), 2.89 (t, *J* = 6.8 Hz, 2H), 2.79 (s, 2H), 1.81 (p, *J* = 6.8 Hz, 2H).

9.2 Synthesis of methyl 4-((3-azidopropyl)carbamoyl)-2-iodobenzoate XZ45



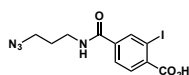
3-Iodo-4-methoxycarbonylbenzoic acid (1.0 g, 3.3 mmol, 1.0 eq.), **XZ44** (491.0 mg, 4.9 mmol, 1.5 eq.), HATU (1.9 g, 4.9 mmol, 1.5 eq.) and DIPEA (1.7 mL, 9.8 mmol, 3.0 eq.) were dissolved in THF (30 mL) and DMF (7 mL). The mixture was stirred at RT for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The volatiles were removed by rotary evaporation and the residue was dissolved in EtOAc (100 mL) and washed with half-saturated NH₄Cl solution (3 x 80 mL) and H₂O (80 mL). The combined aqueous layers were extracted with EtOAc (100 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by flash column chromatography (Silica, 150 g, cyclohexane:EtOAc 2:1, *R_f* = 0.28, UV). Product-containing fractions were combined and concentrated *in vacuo* to yield the desired product as a yellowish oil (1.2 g, **95%**).

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 8.34 (d, *J* = 1.7 Hz, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.77 (dd, *J* = 8.1, 1.7 Hz, 1H), 6.41 (s, 1H), 3.95 (s, 3H), 3.56 (td, *J* = 6.6, 5.8 Hz, 2H), 3.47 (t, *J* = 6.4 Hz, 2H), 1.92 (p, *J* = 6.6 Hz, 2H).

¹³C NMR (101 MHz, CDCl₃) δ/ppm: 166.51, 165.27, 139.77, 138.11, 137.85, 131.11, 126.49, 94.24, 52.91, 49.78, 38.31, 28.77.

HRMS (ESI): C₁₂H₁₄IN₄O₃⁺ *calcd*: 389.0105, *found*: 389.0109.

9.3 Synthesis of 4-((3-azidopropyl)carbamoyl)-2-iodobenzoic acid XZ46



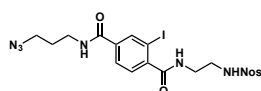
XZ45 (1.2 g, 3.1 mmol, 1.0 eq.) was dissolved in MeCN (10 mL) and a solution of LiOH hydrate (646.0 mg, 15.4 mmol, 5.0 eq.) in H₂O (10 mL) was added. The solution was stirred at RT for 4 h after which UPLC-MS analysis showed complete conversion to the desired product. The reaction mixture was acidified with TFA to pH 2-3 and directly purified by reversed phase flash column chromatography on the ISOLERA (Rp-Silica, 100 g, H₂O/MeCN + 0.1% TFA, UV, 2 runs). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (1.1 g, **98%**).

¹H NMR (400 MHz, DMSO-*d*₆) δ/ppm: 13.54 (s, 1H), 8.72 (t, *J* = 5.6 Hz, 1H), 8.38 (d, *J* = 1.6 Hz, 1H), 7.90 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.76 (d, *J* = 8.0 Hz, 1H), 3.41 (t, *J* = 6.7 Hz, 2H), 3.32 (q, *J* = 6.5 Hz, 2H), 1.78 (p, *J* = 6.8 Hz, 2H).

¹³C NMR (101 MHz, DMSO) δ/ppm: 168.02, 164.17, 138.70, 137.16, 129.56, 126.94, 93.82, 48.50, 36.80, 28.22.

HRMS (ESI): C₁₁H₁₁IN₄NaO₃⁺ *calcd*: 396.9768, *found*: 396.9769.

9.4 Synthesis of (NOSYL)ethyl-4-((3-azidopropyl)carbamoyl)-2-iodobenzoic amide XZ47



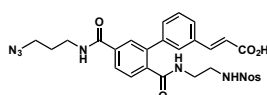
XZ46 (1.1 g, 3.0 mmol, 1.0 eq.), **XZ1** (1.1 g, 4.5 mmol, 1.5 eq.), HATU (1.4 g, 3.6 mmol, 1.2 eq.) and DIPEA (1.6 mL, 9.0 mmol, 3.0 eq.) were dissolved in DMF (12 mL) and stirred at RT for 3 h after which UPLC-MS analysis showed complete conversion of the starting material. The volatiles were removed *in vacuo* and the residue was dissolved in EtOAc (90 mL). The organic layer was washed with saturated NH₄Cl (3 x 80 mL) and H₂O (80 mL) and the combined aqueous layers were extracted with EtOAc (100 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by flash column chromatography (Silica, 180 g, 1:4 cyclohexane:EtOAc, R_f = 0.2, UV) to yield the desired product as a yellow solid (1.6 g, **92%**).

¹H NMR (400 MHz, Acetonitrile-*d*₃) δ/ppm: 8.25 (d, *J* = 1.6 Hz, 1H), 8.08 (dd, *J* = 5.9, 3.4 Hz, 1H), 7.89 – 7.85 (m, 1H), 7.84 – 7.76 (m, 3H), 7.37 (d, *J* = 7.9 Hz, 1H), 7.16 (s, 1H), 6.95 (s, 1H), 6.19 (s, 1H), 3.49 – 3.43 (m, 2H), 3.43 – 3.36 (m, 4H), 3.27 (t, *J* = 6.0 Hz, 2H), 1.83 (t, *J* = 6.7 Hz, 2H).

¹³C NMR (101 MHz, CDCl₃) δ/ppm: 169.91, 166.24, 148.14, 144.06, 138.37, 136.61, 133.90, 133.63, 133.06, 131.15, 127.77, 126.70, 92.40, 49.58, 43.39, 40.13, 39.11, 28.75.

HRMS (ESI): C₁₉H₂₀IN₇NaO₆S⁺ *calcd*: 624.0133, *found*: 624.0144.

9.5 Synthesis of NP01



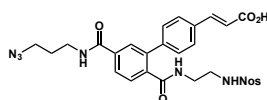
XZ47 (43.0 mg, 71.5 μmol, 1.0 eq.), 3-(2-carboxyvinyl)phenylboronic acid (27.5 mg, 143.0 μmol, 2.0 eq.), Pd (π-cinnamyl) chloride dimer (7.4 mg, 14.3 μmol, 20 mol%) and K₃PO₄ (45.5 mg, 215.0 μmol, 3.0 eq.) were suspended in EtOH (800 μL) and H₂O (400 μL). The mixture was stirred at 50°C for 1 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (28 mg, **63%**).

¹H NMR (500 MHz, DMSO-*d*₆) δ/ppm: 12.43 (s, 1H), 8.65 (t, *J* = 5.7 Hz, 1H), 8.33 (t, *J* = 5.9 Hz, 1H), 8.14 (t, *J* = 6.0 Hz, 1H), 8.00 (dd, *J* = 5.8, 3.3 Hz, 1H), 7.96 – 7.92 (m, 1H), 7.92 – 7.85 (m, 4H), 7.69 – 7.66 (m, 1H), 7.64 – 7.55 (m, 2H), 7.53 (d, *J* = 8.4 Hz, 1H), 7.42 – 7.33 (m, 2H), 6.52 (d, *J* = 16.0 Hz, 1H), 3.41 (t, *J* = 6.7 Hz, 2H), 3.37 – 3.34 (m, 2H), 3.15 (dt, *J* = 7.9, 6.1 Hz, 2H), 2.77 (dt, *J* = 8.2, 6.1 Hz, 2H), 1.79 (p, *J* = 6.8 Hz, 2H).

¹³C NMR (500 MHz, HMBC/HMQC, DMSO-*d*₆) δ/ppm: 166.97, 168.24, 165.11, 147.52, 143.29, 139.92, 139.83, 139.56, 138.75, 138.04, 134.89, 133.88, 132.47, 129.89, 129.09, 128.43, 128.17, 127.70, 127.49, 127.04, 126.05, 124.31, 119.37, 48.22, 41.17, 38.46, 36.42, 28.00.

HRMS (ESI): C₂₈H₂₇N₇NaO₈S⁺ *calcd*: 644.1534, *found*: 644.1534.

9.6 Synthesis of NP02



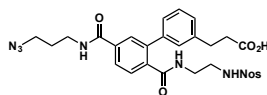
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 4-(2-carboxyvinyl)phenylboronic acid (12.8 mg, 66.5 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (16 mg, **77%**).

^1H NMR (400 MHz, Acetone- d_6) δ /ppm: 8.13 – 8.08 (m, 1H), 8.05 (t, J = 5.7 Hz, 1H), 7.99 – 7.94 (m, 1H), 7.93 – 7.86 (m, 4H), 7.71 – 7.64 (m, 3H), 7.59 (d, J = 8.5 Hz, 1H), 7.55 – 7.45 (m, 3H), 6.75 (t, J = 6.0 Hz, 1H), 6.54 (d, J = 16.1 Hz, 1H), 3.54 – 3.44 (m, 4H), 3.39 (q, J = 6.3 Hz, 2H), 3.13 (q, J = 6.1 Hz, 2H), 1.90 (p, J = 6.7 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, Acetone- d_6) δ /ppm: 169.71, 167.35, 166.37, 148.91, 144.75, 142.56, 142.47, 139.76, 136.73, 134.80, 134.53, 133.85, 133.43, 131.21, 129.91, 129.19, 128.89, 128.79, 127.04, 125.66, 119.28, 49.60, 43.32, 39.89, 37.60, 29.35.

HRMS (ESI): $\text{C}_{28}\text{H}_{27}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 644.1534, *found*: 644.1534.

9.7 Synthesis of NP03



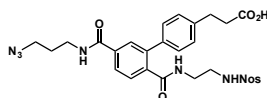
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 3-(2-carboxyethyl)phenylboronic acid (12.9 mg, 66.5 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (15 mg, **72%**).

^1H NMR (500 MHz, Acetone- d_6) δ /ppm: 8.13 – 8.07 (m, 1H), 7.99 – 7.94 (m, 1H), 7.95 – 7.90 (m, 2H), 7.88 (dd, J = 7.9, 1.8 Hz, 1H), 7.86 (dd, J = 1.8, 0.6 Hz, 1H), 7.56 (dd, J = 7.8, 0.5 Hz, 1H), 7.32 – 7.29 (m, 1H), 7.28 (dd, J = 7.1, 0.7 Hz, 1H), 7.26 – 7.21 (m, 2H), 3.48 (dt, J = 15.6, 6.8 Hz, 4H), 3.33 (t, J = 6.5 Hz, 2H), 3.05 (t, J = 6.5 Hz, 2H), 2.91 (t, J = 7.7 Hz, 2H), 2.61 (t, J = 7.7 Hz, 2H), 1.90 (p, J = 6.7 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, Acetone- d_6) δ /ppm: 173.62, 169.79, 166.57, 148.92, 141.85, 141.25, 140.59, 140.40, 139.74, 136.55, 134.74, 133.42, 131.20, 129.31, 129.23, 129.02, 128.74, 128.29, 127.09, 126.57, 125.64, 49.57, 43.17, 39.78, 37.53, 35.51, 31.16, 29.32.

HRMS (ESI): $\text{C}_{28}\text{H}_{29}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 646.1691, *found*: 646.1695.

9.8 Synthesis of NP04



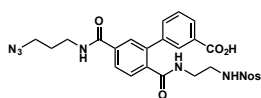
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 4-(2-carboxyethyl)phenylboronic acid (12.9 mg, 66.5 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (14 mg, **68%**).

^1H NMR (500 MHz, Acetone- d_6) δ /ppm: 8.14 – 8.09 (m, 1H), 8.00 – 7.95 (m, 1H), 7.96 – 7.90 (m, 2H), 7.87 (dd, J = 7.9, 1.8 Hz, 1H), 7.84 (d, J = 1.6 Hz, 1H), 7.55 (d, J = 7.9 Hz, 1H), 7.37 – 7.30 (m, 2H), 7.29 – 7.23 (m, 2H), 3.48 (dt, J = 15.9, 6.8 Hz, 4H), 3.34 (t, J = 6.3 Hz, 2H), 3.09 (t, J = 6.3 Hz, 2H), 2.91 (t, J = 7.7 Hz, 2H), 2.62 (t, J = 7.7 Hz, 2H), 1.90 (p, J = 6.7 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, Acetone- d_6) δ /ppm: 173.60, 169.93, 166.46, 148.91, 141.23, 140.33, 140.22, 139.62, 138.36, 136.49, 134.78, 133.43, 131.22, 129.31, 129.19, 128.97, 128.81, 126.56, 125.66, 49.58, 43.30, 39.84, 37.56, 35.45, 30.89, 29.33.

HRMS (ESI): $\text{C}_{28}\text{H}_{29}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 646.1691, *found*: 646.1692.

9.9 Synthesis of NP05



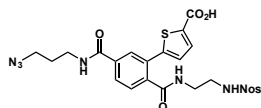
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 3-carboxyphenylboronic acid (12.9 mg, 77.8 μmol , 2.3 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (16 mg, **81%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.70 (t, J = 5.7 Hz, 1H), 8.39 (t, J = 5.8 Hz, 1H), 8.13 (t, J = 6.0 Hz, 1H), 8.02 – 7.97 (m, 2H), 7.97 – 7.93 (m, 1H), 7.92 – 7.85 (m, 5H), 7.61 (ddd, J = 7.6, 1.9, 1.2 Hz, 1H), 7.54 (d, J = 7.8 Hz, 1H), 7.46 (t, J = 7.7 Hz, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.34 (td, J = 6.8, 5.5 Hz, 2H), 3.14 (dt, J = 7.9, 6.0 Hz, 2H), 2.82 (dt, J = 8.3, 6.1 Hz, 2H), 1.79 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 168.29, 166.84, 165.12, 147.44, 139.64, 139.44, 138.90, 137.93, 135.09, 133.92, 132.57, 132.52, 130.52, 129.16, 129.04, 128.25, 128.14, 128.11, 127.69, 126.23, 124.35, 48.28, 41.25, 38.56, 36.48, 28.05.

HRMS (ESI): $\text{C}_{26}\text{H}_{25}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 618.1378, *found*: 618.1381.

9.10 Synthesis of NP06



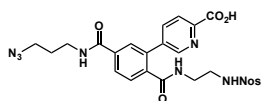
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 5-carboxythiophene-2-boronic acid pinacol ester (16.9 mg, 66.5 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (2 mg, **10%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.72 (t, J = 5.6 Hz, 1H), 8.59 (t, J = 5.8 Hz, 1H), 8.18 (t, J = 6.0 Hz, 1H), 8.03 – 7.94 (m, 3H), 7.92 – 7.85 (m, 3H), 7.64 (d, J = 3.9 Hz, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.26 (d, J = 3.9 Hz, 1H), 3.44 – 3.40 (m, 2H), 3.37 – 3.32 (m, 2H), 3.24 (dt, J = 7.4, 6.1 Hz, 2H), 2.98 (dt, J = 7.8, 6.1 Hz, 2H), 1.80 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 168.00, 164.76, 162.44, 147.44, 146.74, 138.67, 135.06, 134.38, 134.00, 133.23, 132.61, 132.20, 130.16, 129.27, 128.09, 127.96, 127.55, 127.15, 124.38, 48.33, 41.45, 38.75, 36.56, 28.06.

HRMS (ESI): $\text{C}_{24}\text{H}_{22}\text{N}_7\text{O}_8\text{S}_2^-$ *calcd*: 600.0977, *found*: 600.0985.

9.11 Synthesis of NP07



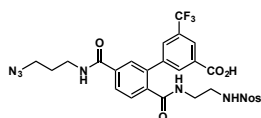
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 2-methoxycarbonylpyridine-5-boronic acid (16.9 mg, 93.4 μmol , 2.8 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 2 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the impure methyl ester of the product as beige solid (7.0 mg, 35%). The material was dissolved in MeCN (0.5 mL) and a solution of LiOH monohydrate (4.8 mg, 115.0 μmol , 10.0 eq.) in H_2O (382 μL) was added. The solution was stirred at RT for 2 h after which UPLC-MS analysis showed full conversion to the desired product. The reaction mixture was purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (6.0 mg, **88%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.75 – 8.66 (m, 2H), 8.57 (t, J = 5.8 Hz, 1H), 8.18 (t, J = 6.0 Hz, 1H), 8.06 (dd, J = 8.0, 0.8 Hz, 1H), 8.02 – 7.92 (m, 5H), 7.91 – 7.84 (m, 2H), 7.64 (d, J = 7.9 Hz, 1H), 3.42 (t, J = 6.8 Hz, 2H), 3.35 (td, J = 6.8, 5.5 Hz, 2H), 3.19 (q, J = 6.4 Hz, 2H), 2.92 (dt, J = 7.5, 6.1 Hz, 2H), 1.80 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 167.81, 165.73, 165.03, 148.41, 147.44, 146.84, 138.34, 138.27, 136.70, 135.24, 134.97, 133.92, 132.56, 132.30, 129.23, 128.58, 128.06, 127.04, 124.32, 123.98, 48.26, 41.43, 38.76, 36.50, 28.03.

HRMS (ESI): $\text{C}_{25}\text{H}_{23}\text{N}_8\text{O}_8\text{S}^-$ *calcd*: 595.1365, *found*: 595.1371.

9.12 Synthesis of NP08



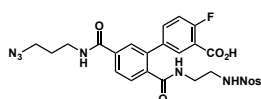
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 3-methoxycarbonyl-5-trifluoromethylphenylboronic acid pinacol ester (22.0 mg, 66.5 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 1 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the impure methyl ester of the product (12.0 mg, 53%). The material was dissolved in MeCN (0.5 mL) and a solution of LiOH monohydrate (7.4 mg, 177.0 μmol , 10.0 eq.) in H_2O (590 μL) was added. The solution was stirred at RT for 2 h after which UPLC-MS analysis showed full conversion to the desired product. The reaction mixture was purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (8.0 mg, **68%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 13.62 (s, 1H), 8.72 (t, J = 5.7 Hz, 1H), 8.51 (t, J = 5.9 Hz, 1H), 8.24 – 8.20 (m, 1H), 8.17 (t, J = 6.0 Hz, 1H), 8.14 – 8.10 (m, 1H), 8.00 – 7.96 (m, 1H), 7.96 – 7.90 (m, 4H), 7.90 – 7.84 (m, 2H), 7.60 (d, J = 7.9 Hz, 1H), 3.42 (t, J = 6.7 Hz, 2H), 3.35 (td, J = 6.9, 5.6 Hz, 2H), 3.14 (dt, J = 8.0, 6.1 Hz, 2H), 2.81 (dt, J = 8.4, 6.0 Hz, 2H), 1.80 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 167.87, 165.38, 164.91, 147.22, 140.92, 138.64, 136.23, 135.33, 133.85, 132.77, 132.44, 129.00, 128.97, 128.47, 128.05, 127.79, 126.97, 124.30, 124.27, 122.17, 120.00, 48.23, 41.00, 38.61, 36.44, 28.02.

HRMS (ESI): $\text{C}_{27}\text{H}_{24}\text{F}_3\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 686.1251, *found*: 686.1262.

9.13 Synthesis of NP09



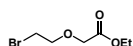
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), 3-carboxy-4-fluorophenylboronic acid (12.2 mg, 66.5 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K_3PO_4 (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H_2O (200 μL). The mixture was stirred at 50°C for 1 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as brownish solid (18.0 mg, **88%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 13.32 (s, 1H), 8.70 (t, J = 5.6 Hz, 1H), 8.44 (t, J = 5.8 Hz, 1H), 8.15 (t, J = 6.0 Hz, 1H), 8.01 – 7.95 (m, 2H), 7.92 – 7.83 (m, 5H), 7.61 (ddd, J = 8.5, 4.5, 2.5 Hz, 1H), 7.54 (d, J = 7.9 Hz, 1H), 7.30 (dd, J = 10.7, 8.5 Hz, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.34 (q, J = 6.6 Hz, 2H), 3.16 (dt, J = 7.5, 6.0 Hz, 2H), 2.88 (dt, J = 8.1, 6.1 Hz, 2H), 1.79 (p, J = 6.8 Hz, 2H).

^{13}C NMR (126 MHz, DMSO) δ /ppm: 168.44, 165.38, 164.84, 159.62, 147.66, 138.93, 137.17, 135.84, 135.41, 134.50, 134.11, 132.73, 132.60, 131.72, 129.37, 128.33, 127.94, 126.50, 124.54, 119.07, 116.94, 116.76, 48.54, 41.61, 38.89, 36.75, 28.34.

HRMS (ESI): $\text{C}_{26}\text{H}_{24}\text{FN}_7\text{NaO}_8\text{S}^+$ *calcd*: 636.1283, *found*: 636.1291.

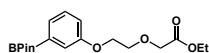
9.14 Synthesis of Ethyl 2-bromoethoxyacetate XZ48



Under an inert atmosphere 2-bromoethanol (709.0 μL , 10.0 mmol, 1.0 eq.) was dissolved in DCM (17 mL) and cooled in an ice bath. $\text{Rh}_2(\text{OAc})_4$ (44.2 mg, 100 μmol , 1.0 mol%) was added and the mixture was stirred for 5 min. The ice bath was removed and a solution of ethyl 2-diazoacetate (1.2 mL, 9.6 mmol, 0.96 eq.) in DCM (8 mL) was added dropwise while gas evolution occurred. The green mixture was stirred at RT for 2 h. The mixture was filtered over Celite and concentrated *in vacuo*. The crude product was purified by bulb-to-bulb distillation (80°C, 3.6×10^{-1} mbar) to yield the desired product as a colorless liquid (1.3 g, **61%**). Analytical data was in agreement with reported data.^[14]

^1H NMR (400 MHz, Chloroform-*d*) δ /ppm: 4.23 (q, J = 7.2 Hz, 2H), 4.15 (s, 2H), 3.89 (t, J = 6.2 Hz, 2H), 3.51 (t, J = 6.2 Hz, 2H), 1.29 (t, J = 7.1 Hz, 3H).

9.15 Synthesis of 3-Ethylloxycarbonylmethoxyethoxyphenylboronic acid pinacol ester XZ49



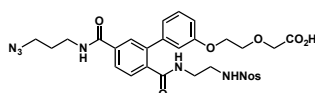
Under an inert atmosphere 3-hydroxyphenylboronic acid pinacol ester (47.0 mg, 214.0 μmol , 1.0 eq.), **XZ48** (54.1 mg, 256.0 μmol , 1.2 eq.) and potassium carbonate (88.5 mg, 641.0 μmol , 3.0 eq.) were mixed in MeCN (1 mL). The suspension was stirred at 65°C for 39 h after which UPLC-MS analysis showed 78% conversion to the desired product. Potassium carbonate (29.5 mg, 213.4 μmol , 1.0 eq.) and **XZ48** (10.8 mg, 51.2 μmol , 0.2 eq.) were added and the mixture was continued stirring at 65°C for another 24 h. UPLC-MS analysis showed >95% conversion to the desired product. The solvent was removed by rotary evaporation and the residue was taken up in DCM (5 mL) and H₂O (5 mL). The organic layer was separated and the aqueous layer was extracted with DCM (2 x 5 mL). The combined organic layers were washed with brine (10 mL), dried over Na₂SO₄ and concentrated *in vacuo*. The residue was purified by flash column chromatography (Silica, 9 g, 10:1→5:1 cyclohexane: EtOAc, R_f = 0.29, KMnO₄) to yield the desired product as a colourless oil (53 mg, **71%**).

¹H NMR (400 MHz, Chloroform-*d*) δ /ppm: 7.40 (dt, *J* = 7.2, 1.0 Hz, 1H), 7.33 (dd, *J* = 2.8, 0.9 Hz, 1H), 7.28 (dd, *J* = 8.3, 7.3 Hz, 1H), 7.03 (ddd, *J* = 8.2, 2.8, 1.1 Hz, 1H), 4.24 – 4.18 (m, 6H), 3.97 – 3.91 (m, 2H), 1.34 (s, 12H), 1.28 (t, *J* = 7.1 Hz, 3H).

¹³C NMR (101 MHz, CDCl₃) δ /ppm: 170.57, 158.18, 129.11, 127.62, 119.72, 118.53, 83.98, 70.27, 69.09, 67.66, 61.04, 25.01, 14.35.

HRMS (ESI): C₁₈H₂₇BNaO₆⁺ *calcd*: 373.1793, *found*: 373.1793.

9.16 Synthesis of NP10



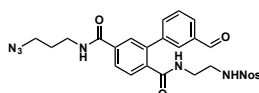
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.), **XZ49** (23.3 mg, 66.5 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (3.5 mg, 6.7 μmol , 20 mol%) and K₃PO₄ (21.2 mg, 99.8 μmol , 3.0 eq.) were suspended in EtOH (400 μL) and H₂O (200 μL). The mixture was stirred at 50°C for 1 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the product as an ester/acid mixture (12.0 mg, 52%). The material was dissolved in MeCN (0.5 mL) and a solution of LiOH monohydrate (7.2 mg, 177.0 μmol , 10.0 eq.) in H₂O (573 μL) was added. The solution was stirred at RT for 2 h after which UPLC-MS analysis showed full conversion to the desired product. The reaction mixture was purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (8.0 mg, **70%**).

¹H NMR (500 MHz, DMSO-*d*₆) δ /ppm: 8.66 (t, *J* = 5.7 Hz, 1H), 8.24 (t, *J* = 5.9 Hz, 1H), 8.13 (t, *J* = 6.0 Hz, 1H), 8.03 – 7.98 (m, 1H), 7.98 – 7.94 (m, 1H), 7.93 – 7.87 (m, 2H), 7.86 – 7.82 (m, 2H), 7.49 (d, *J* = 8.4 Hz, 1H), 7.20 (t, *J* = 7.9 Hz, 1H), 6.98 (dd, *J* = 2.6, 1.6 Hz, 1H), 6.95 – 6.91 (m, 1H), 6.86 (ddd, *J* = 8.3, 2.6, 0.9 Hz, 1H), 4.12 – 4.06 (m, 4H), 3.82 – 3.79 (m, 2H), 3.41 (t, *J* = 6.7 Hz, 2H), 3.34 (q, *J* = 6.5 Hz, 2H), 3.15 (dt, *J* = 8.0, 6.1 Hz, 2H), 2.79 (dt, *J* = 8.3, 6.1 Hz, 2H), 1.79 (p, *J* = 6.8 Hz, 2H).

¹³C NMR (500 MHz, HMBC/HMQC, DMSO-*d*₆) δ /ppm: 171.35, 168.53, 165.26, 157.94, 147.49, 140.71, 138.87, 138.56, 134.89, 133.95, 132.60, 132.28, 129.20, 128.99, 128.06, 127.52, 125.95, 124.37, 120.64, 114.61, 113.04, 68.76, 67.38, 66.83, 48.29, 41.25, 38.50, 36.48, 28.06.

HRMS (ESI): C₂₉H₃₁N₇NaO₁₀S⁺ *calcd*: 692.1745, *found*: 692.1751.

9.17 Synthesis of *N*⁵-(3-azidopropyl)-3'-formyl-*N*²-(2-NOSYL-ethyl)-[1,1'-biphenyl]-2,5-dicarboxamide **XZ50**



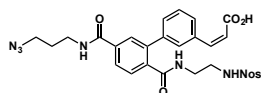
XZ47 (53.0 mg, 88.1 μmol , 1.0 eq.), 3-formylphenylboronic acid (26.4 mg, 176.0 μmol , 2.0 eq.), Pd (π -cinnamyl) chloride dimer (9.1 mg, 17.6 μmol , 20 mol%) and K₃PO₄ (56.1 mg, 264.0 μmol , 3.0 eq.) were suspended in EtOH (1 mL) and H₂O (500 μL). The mixture was stirred at 50°C for 1 h after which UPLC-MS analysis showed complete conversion to the desired product. The black mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as white solid (34.0 mg, **67%**).

¹H NMR (400 MHz, Chloroform-*d*) δ /ppm: 9.89 (s, 1H), 8.13 – 7.98 (m, 1H), 7.88 – 7.81 (m, 1H), 7.80 – 7.66 (m, 4H), 7.58 (d, *J* = 1.6 Hz, 1H), 7.55 – 7.39 (m, 3H), 7.38 – 7.27 (m, 3H), 6.08 (t, *J* = 5.9 Hz, 1H), 3.50 (q, *J* = 6.4 Hz, 2H), 3.43 (t, *J* = 6.5 Hz, 2H), 3.33 (q, *J* = 5.5 Hz, 2H), 3.06 (t, *J* = 5.6 Hz, 2H), 1.89 (p, *J* = 6.7 Hz, 2H).

¹³C NMR (101 MHz, CDCl₃) δ /ppm: 192.55, 169.98, 167.53, 148.08, 140.04, 138.72, 137.89, 136.48, 136.01, 134.64, 133.92, 133.36, 132.97, 131.06, 130.20, 129.50, 128.86, 128.79, 128.41, 126.34, 125.44, 49.52, 42.97, 39.97, 38.15, 28.64.

HRMS (ESI): C₂₆H₂₅N₇NaO₇S⁺ *calcd*: 602.1428, *found*: 602.1438.

9.18 Synthesis of NP11



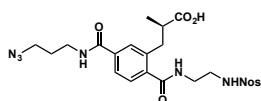
Under an inert atmosphere 18-crown-6 ether (3.0 mg, 11.2 μmol , 1.3 eq.) was dissolved in THF (500 μL) and KHMDS (2.1 mg, 10.4 μmol , 1.2 eq.) was added. The mixture was cooled to -78°C in a dry ice/acetone bath. Methyl 2-[bis(2,2,2-trifluoroethoxy)phosphoryl]acetate (2.0 μL , 9.5 μmol , 1.1 eq.) was added and the mixture was stirred at -78°C for 5 min. **XZ50** (5.0 mg, 8.6 μmol , 1.0 eq.) was added and the mixture was stirred at -78°C for 3 h. The cooling bath was removed and the solution was slowly warmed to RT. After in total 6 h reaction time UPLC-MS analysis showed full conversion to the methyl ester product. A solution of LiOH monohydrate (3.6 mg, 86.3 μmol , 10.0 eq.) in H_2O (288 μL) was added and the mixture was continued stirring overnight. UPLC-MS analysis showed full conversion to the acid in a 3:1 isomeric ratio. The mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (2.5 mg, **47%**, 24:1 *cis:trans* mixture).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 12.51 (s, 1H), 8.66 (t, J = 5.6 Hz, 1H), 8.32 (t, J = 5.9 Hz, 1H), 8.15 (t, J = 6.0 Hz, 1H), 8.03 – 7.99 (m, 1H), 7.98 – 7.94 (m, 1H), 7.92 – 7.88 (m, 2H), 7.87 – 7.83 (m, 2H), 7.59 – 7.55 (m, 2H), 7.51 (d, J = 7.8 Hz, 1H), 7.37 – 7.27 (m, 2H), 6.85 (d, J = 12.7 Hz, 1H), 5.94 (d, J = 12.7 Hz, 1H), 3.45 – 3.41 (m, 2H), 3.34 (q, J = 6.2 Hz, 2H), 3.16 (dt, J = 7.9, 6.0 Hz, 2H), 2.81 (dt, J = 8.2, 6.1 Hz, 2H), 1.79 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 168.31, 167.00, 165.26, 147.32, 139.93, 139.23, 138.75, 138.51, 135.11, 134.55, 133.96, 132.59, 130.73, 129.51, 129.19, 128.61, 128.28, 128.18, 127.64, 127.62, 125.90, 124.37, 121.19, 48.30, 41.28, 38.58, 36.50, 28.06.

HRMS (ESI): $\text{C}_{28}\text{H}_{27}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 644.1534, *found*: 644.1540.

9.19 Synthesis of NP12



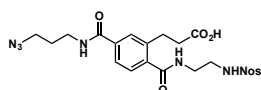
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.) and Pd (π -cinnamyl) chloride dimer (1.7 mg, 3.3 μmol , 10 mol%) were placed in a dry 5 mL Schlenk tube, which was evacuated and backfilled with N_2 three times. THF (400 μL) was added and the yellow solution was cooled to 0°C . A solution of (*S*)-(3-methoxy-2-methyl-3-oxopropyl)zinc(II) bromide (99.8 μL , 49.9 μmol , 500 mM in THF, 1.5 eq.) was slowly added and the yellow solution was stirred at 0°C for 15 min. The solution was warmed to RT and stirred for another 45 min. UPLC-MS analysis showed full conversion to the methyl ester product. A solution of LiOH monohydrate (7.0 mg, 166.0 μmol , 5.0 eq.) in H_2O (333 μL) was added and the mixture was stirred overnight. UPLC-MS analysis showed complete conversion to the desired product. The mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (15 mg, **80%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.55 (t, J = 5.7 Hz, 1H), 8.44 (t, J = 5.7 Hz, 1H), 8.20 (t, J = 5.9 Hz, 1H), 8.04 – 8.00 (m, 1H), 8.00 – 7.96 (m, 1H), 7.90 – 7.85 (m, 2H), 7.73 – 7.68 (m, 2H), 7.44 – 7.40 (m, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.32 (qd, J = 6.2, 3.0 Hz, 4H), 3.11 – 3.01 (m, 3H), 2.76 (dd, J = 13.5, 7.8 Hz, 1H), 2.65 (h, J = 7.1 Hz, 1H), 1.79 (p, J = 6.8 Hz, 2H), 0.97 (d, J = 7.0 Hz, 3H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 176.50, 168.42, 165.38, 147.43, 138.92, 137.58, 137.34, 134.63, 133.88, 132.52, 129.23, 128.87, 127.14, 124.57, 124.28, 48.26, 41.67, 39.92, 38.74, 36.41, 35.68, 28.05, 16.50.

HRMS (ESI): $\text{C}_{23}\text{H}_{27}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 584.1534, *found*: 584.1542.

9.20 Synthesis of NP13



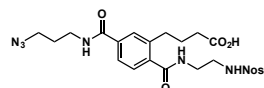
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.) and Pd (π -cinnamyl) chloride dimer (1.7 mg, 3.3 μmol , 10 mol%) were placed in a dry 5 mL Schlenk tube, which was evacuated and backfilled with N_2 three times. THF (400 μL) was added and the yellow solution was cooled to 0°C . A solution of (3-ethoxy-3-oxopropyl)zinc(II) bromide (99.8 μL , 49.9 μmol , 500 mM in THF, 1.5 eq.) was slowly added and the yellow solution was stirred at 0°C for 15 min. The solution was warmed to RT and stirred for another 45 min. UPLC-MS analysis showed full conversion to the ethyl ester product. A solution of LiOH monohydrate (14.0 mg, 333.0 μmol , 10.0 eq.) in H_2O (665 μL) was added and the mixture was stirred at RT for 2 h. UPLC-MS analysis showed complete conversion to the desired product. The mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (16 mg, **88%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.55 (t, J = 5.7 Hz, 1H), 8.44 (t, J = 5.7 Hz, 1H), 8.22 (t, J = 6.0 Hz, 1H), 8.05 – 8.00 (m, 1H), 8.00 – 7.96 (m, 1H), 7.90 – 7.84 (m, 2H), 7.75 (d, J = 1.7 Hz, 1H), 7.69 (dd, J = 7.9, 1.8 Hz, 1H), 7.41 (d, J = 7.9 Hz, 1H), 3.41 (t, J = 6.8 Hz, 2H), 3.35 – 3.29 (m, 4H), 3.09 (q, J = 6.5 Hz, 2H), 2.91 (dd, J = 8.7, 7.1 Hz, 2H), 2.53 (dd, J = 8.7, 7.2 Hz, 2H), 1.79 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 173.51, 168.47, 165.50, 147.47, 138.64, 138.57, 135.03, 133.96, 132.63, 129.31, 128.31, 127.17, 126.62, 124.53, 124.38, 48.35, 41.79, 38.86, 36.48, 34.97, 28.15, 27.89.

HRMS (ESI): $\text{C}_{22}\text{H}_{25}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 570.1378, *found*: 570.1385.

9.21 Synthesis of NP14



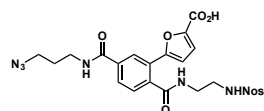
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.) and Pd (π -cinnamyl) chloride dimer (1.7 mg, 3.3 μmol , 10 mol%) were placed in a dry 5 mL Schlenk tube, which was evacuated and backfilled with N_2 three times. THF (400 μL) was added and the yellow solution was cooled to 0°C . A solution of (4-ethoxy-4-oxobutyl)zinc(II) bromide (99.8 μL , 49.9 μmol , 500 mM in THF, 1.5 eq.) was slowly added and the yellow solution was stirred at 0°C for 15 min. The solution was warmed to RT and stirred for another 45 min. UPLC-MS analysis showed full conversion to the ethyl ester product. A solution of LiOH monohydrate (14.0 mg, 333.0 μmol , 10.0 eq.) in H_2O (665 μL) was added and the mixture was stirred at RT for 2 h. UPLC-MS analysis showed complete conversion to the desired product. The mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (7 mg, **38%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.57 (t, J = 5.7 Hz, 1H), 8.40 (t, J = 5.7 Hz, 1H), 8.20 (t, J = 5.9 Hz, 1H), 8.06 – 8.00 (m, 1H), 8.01 – 7.96 (m, 1H), 7.90 – 7.84 (m, 2H), 7.71 (d, J = 1.7 Hz, 1H), 7.69 (dd, J = 7.9, 1.7 Hz, 1H), 7.38 (d, J = 7.9 Hz, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.32 (p, J = 6.3 Hz, 4H), 3.08 (q, J = 6.4 Hz, 2H), 2.73 – 2.67 (m, 2H), 2.19 (t, J = 7.5 Hz, 2H), 1.78 (dt, J = 10.2, 7.1 Hz, 4H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 173.93, 168.52, 165.48, 147.44, 139.23, 139.02, 138.80, 134.82, 133.91, 132.56, 129.27, 128.17, 127.01, 124.34, 124.30, 48.31, 41.77, 38.75, 36.44, 33.13, 31.63, 28.11, 25.95.

HRMS (ESI): $\text{C}_{23}\text{H}_{27}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 584.1534, *found*: 584.1533.

9.22 Synthesis of NP15



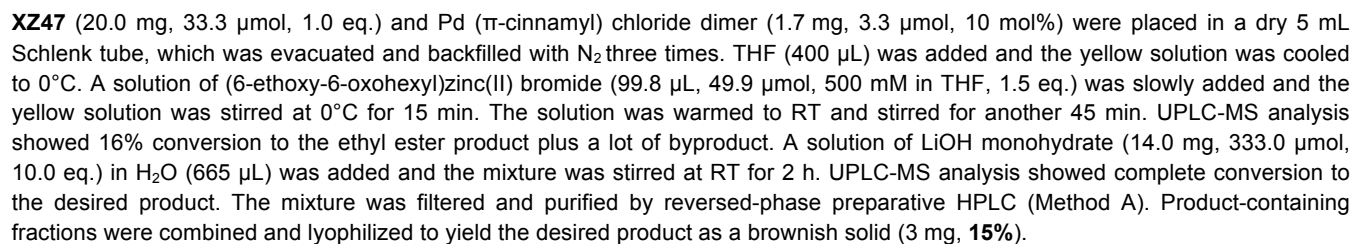
XZ47 (20.0 mg, 33.3 μmol , 1.0 eq.) and Pd (π -cinnamyl) chloride dimer (6.7 mg, 13.3 μmol , 40 mol%) were placed in a dry 5 mL Schlenk tube, which was evacuated and backfilled with N_2 three times. THF (400 μL) was added and the yellow solution was cooled to 0°C . A solution of (5-ethoxycarbonyl-2-furyl)zinc(II) bromide (99.8 μL , 49.9 μmol , 500 mM in THF, 1.5 eq.) was slowly added and the yellow solution was stirred at 0°C for 15 min. The solution was warmed to RT and stirred for another 45 min. UPLC-MS analysis showed 50% conversion to the ethyl ester product. The mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a yellow-brown solid (6 mg, 29%). The material was dissolved in MeCN (300 μL) and a solution of LiOH monohydrate (4.1 mg, 97.8 μmol , 10.0 eq.) in H_2O (196 μL) was added and the mixture was stirred at RT for 2 h. UPLC-MS analysis showed complete conversion to the desired product. The mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (3 mg, **52%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.74 (t, J = 5.6 Hz, 1H), 8.60 (t, J = 5.7 Hz, 1H), 8.23 – 8.18 (m, 2H), 8.04 – 8.01 (m, 1H), 8.01 – 7.97 (m, 1H), 7.91 – 7.85 (m, 3H), 7.50 (d, J = 7.9 Hz, 1H), 7.25 (d, J = 3.6 Hz, 1H), 6.85 (d, J = 3.6 Hz, 1H), 3.43 (t, J = 6.7 Hz, 2H), 3.34 (dq, J = 19.1, 6.6 Hz, 4H), 3.08 (dt, J = 7.6, 6.2 Hz, 2H), 1.81 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 168.20, 164.92, 153.68, 147.43, 144.15, 137.36, 135.03, 133.89, 132.57, 129.22, 127.92, 126.99, 126.00, 125.59, 124.30, 119.27, 110.50, 99.05, 48.27, 41.54, 38.86, 36.52, 28.03.

HRMS (ESI): $\text{C}_{24}\text{H}_{23}\text{N}_7\text{NaO}_9\text{S}^+$ *calcd*: 608.1170, *found*: 608.1167.

9.23 Synthesis of NP16

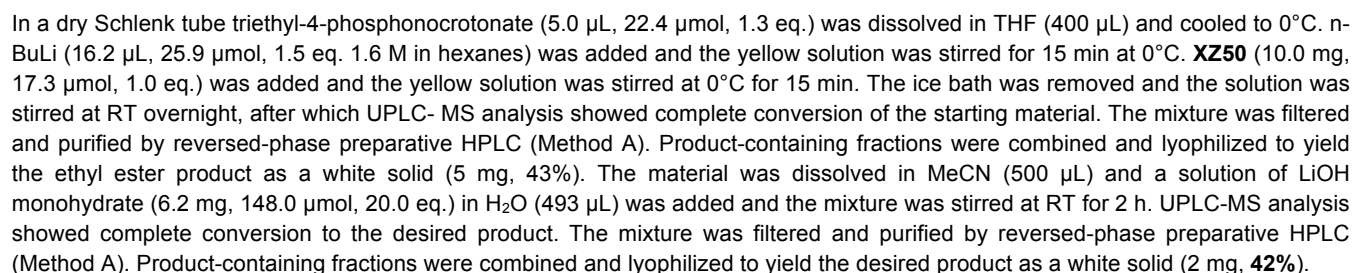


¹H NMR (500 MHz, DMSO-*d*₆) δ/ppm: 11.96 (s, 1H), 8.54 (t, *J* = 5.7 Hz, 1H), 8.38 (t, *J* = 5.7 Hz, 1H), 8.20 (t, *J* = 6.0 Hz, 1H), 8.04 – 8.00 (m, 1H), 8.00 – 7.96 (m, 1H), 7.91 – 7.85 (m, 2H), 7.70 (d, *J* = 1.7 Hz, 1H), 7.67 (dd, *J* = 7.9, 1.8 Hz, 1H), 7.37 (d, *J* = 7.9 Hz, 1H), 3.41 (t, *J* = 6.7 Hz, 2H), 3.33 – 3.28 (m, 4H), 3.10 – 3.03 (m, 2H), 2.72 – 2.64 (m, 2H), 2.16 (t, *J* = 7.4 Hz, 2H), 1.78 (p, *J* = 6.8 Hz, 2H), 1.56 – 1.42 (m, 4H), 1.32 – 1.20 (m, 2H).

¹³C NMR (500 MHz, HMBC/HMQC, DMSO-*d*₆) δ/ppm: 174.21, 168.64, 165.59, 147.46, 139.96, 138.83, 138.74, 134.79, 133.92, 132.55, 129.16, 128.17, 126.95, 124.31, 124.16, 48.32, 41.76, 38.70, 36.44, 33.29, 32.21, 30.45, 28.25, 28.11, 23.99.

HRMS (ESI): $C_{25}H_{31}N_7NaO_8S^+$ *calcd*: 612.1847, *found*: 612.1846.

9.24 Synthesis of NP17

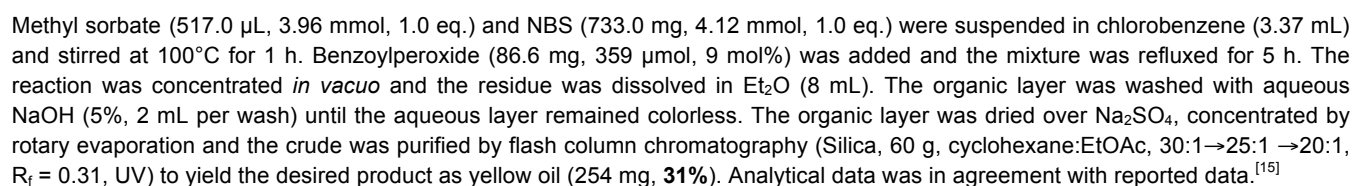


¹H NMR (500 MHz, DMSO-*d*₆) δ/ppm: 12.29 (brs, 1 H), 8.66 (t, *J* = 5.7 Hz, 1H), 8.31 (t, *J* = 5.9 Hz, 1H), 8.14 (t, *J* = 6.0 Hz, 1H), 8.02 – 7.97 (m, 1H), 7.96 – 7.92 (m, Hz, 1H), 7.91 – 7.88 (m, 2H), 7.88 – 7.85 (m, 2H), 7.57 – 7.51 (m, 2H), 7.48 (dt, *J* = 7.2, 1.8 Hz, 1H), 7.38 – 7.28 (m, 3H), 7.14 – 7.00 (m, 2H), 6.01 (d, *J* = 15.2 Hz, 1H), 3.41 (t, *J* = 6.8 Hz, 2H), 3.34 – 3.31 (m, 2 H), 3.15 (dt, *J* = 8.0, 6.1 Hz, 2H), 2.76 (dt, *J* = 8.4, 6.2 Hz, 2H), 1.79 (p, *J* = 6.8 Hz, 2H).

¹³C NMR (500 MHz, HMBC/HMQC, DMSO-*d*₆) δ/ppm: 168.31, 167.23, 165.19, 147.47, 144.03, 143.89, 139.95, 139.31, 138.81, 138.39, 135.71, 135.00, 133.95, 132.56, 129.17, 128.63, 128.36, 128.19, 127.54, 126.95, 126.79, 126.04, 125.97, 124.38, 122.26, 48.30, 41.24, 38.53, 36.50, 28.07.

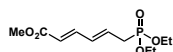
HRMS (ESI): $C_{30}H_{29}N_7NaO_8S^+$ *calcd*: 670.1691, *found*: 670.1694.

9.25 Synthesis of 6-Bromo methyl sorbate XZ51



¹H NMR (400 MHz, CDCl₃) δ/ppm: 7.29 – 7.23 (m, 1H), 6.42 – 6.35 (m, 1H), 6.28 – 6.20 (m, 1H), 5.94 (d, *J* = 15.2 Hz, 1H), 4.03 (d, *J* = 7.7 Hz, 2H), 3.75 (s, 3H).

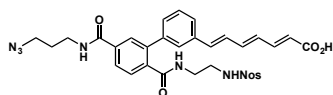
9.26 Synthesis of Methyl (2*E*,4*E*)-6-(diethoxyphosphoryl)hexa-2,4-dienoate XZ52



XZ51 (100.0 mg, 488.0 μmol , 1.0 eq.) was dissolved in toluene (935 μL) and $\text{P}(\text{OEt})_3$ (1.04 mL, 5.9 mmol, 12 eq.) was dropwise added. The mixture was refluxed for 2 h after which the solvent was removed by rotary evaporation. The crude residue was purified by flash column chromatography (Silica, 15 g, petrol ether:EtOAc, 1:1 \rightarrow 1:4 \rightarrow 100% EtOAc, R_f = 0.25, UV) to yield the desired product as a colorless oil (104 mg, **81%**). Analytical data was in agreement with reported data.^[16]

^1H NMR (400 MHz, CDCl_3) δ /ppm: 7.29 – 7.22 (m, 1H), 6.34 – 6.26 (m, 1H), 6.10 – 6.00 (m, 1H), 5.85 (dd, J = 15.4, 2.4 Hz, 1H), 4.14 – 4.06 (m, 4H), 3.74 (s, 3H), 2.71 (dd, J = 23, 7.6 Hz, 2H), 1.31 (t, J = 7.1 Hz, 3H).

9.27 Synthesis of NP18



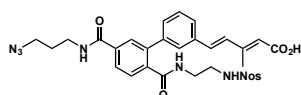
XZ52 (10.9 mg, 41.4 μmol , 1.2 eq.) was dissolved in THF (600 μL) and cooled to -78°C . $n\text{-BuLi}$ (32.4 μL , 51.8 μmol , 1.5 eq. 1.6 M in hexanes) was added and stirred at -78°C for 15 min. A solution of **XZ50** (20.0 mg, 34.5 μmol , 1.0 eq.) in THF (400 μL) was added and the mixture was stirred at -78°C for 2 h. The mixture was slowly warmed to RT and continued stirring for 5.5 h after which HMPA (14.4 μL , 82.8 μmol , 2.4 eq.) was added. The mixture was stirred at RT overnight, filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a yellowish solid (14 mg, 59%). The material was dissolved in MeCN (400 μL) and a solution of LiOH monohydrate (8.5 mg, 203.7 μmol , 10 eq.) in H_2O (310 μL) was added. The mixture was agitated at RT for 1 h, after which UPLC-MS analysis showed complete conversion to the desired product. The solution was acidified to pH 3 with aqueous HCl. The crude was purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a yellowish solid (2.6 mg, 24%).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 12.26 (s, 1H), 8.70 – 8.61 (m, 1H), 8.30 (td, J = 5.8, 3.3 Hz, 1H), 8.17 – 8.09 (m, 1H), 8.03 – 7.97 (m, 1H), 7.96 – 7.92 (m, 1H), 7.92 – 7.81 (m, 4H), 7.67 – 7.43 (m, 3H), 7.42 – 7.24 (m, 3H), 7.23 – 6.99 (m, 1H), 6.90 – 6.72 (m, 1H), 6.62 – 6.45 (m, 1H), 6.40 – 6.13 (m, 1H), 5.95 (dd, J = 17.3, 15.1 Hz, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.34 – 3.31 (m, 2H), 3.15 (q, J = 6.7 Hz, 2H), 2.83 – 2.71 (m, 2H), 1.79 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 168.42, 167.36, 165.25, 147.44, 139.95, 138.88, 138.53, 136.81, 136.26, 135.83, 135.01, 133.94, 132.58, 130.62, 129.19, 128.61, 128.22, 128.20, 127.53, 127.08, 127.00, 126.51, 125.99, 125.95, 125.73, 124.38, 121.67, 48.31, 41.27, 38.55, 36.51, 28.07.

HRMS (ESI): $\text{C}_{32}\text{H}_{31}\text{N}_7\text{NaO}_8\text{S}^+$ *calcd*: 696.1847, *found*: 696.1855.

9.28 Synthesis of NP19



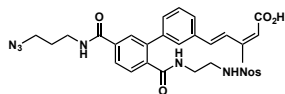
In a dry Schlenk tube triethyl 3-methyl-4-phosphono-2-butenolate (24.2 μL , 89.8 μmol , 1.3 eq.) was dissolved in THF (1.6 mL) and cooled to 0°C . $n\text{-BuLi}$ (64.8 μL , 103.6 μmol , 1.5 eq. 1.6 M in hexanes) was added and the yellow solution was stirred at 0°C for 15 min. **XZ50** (40.0 mg, 69.0 μmol , 1.0 eq.) was added and the yellow solution was stirred at 0°C for 15 min. The ice bath was removed and the solution was stirred at RT for 38 h after which UPLC-MS analysis showed about 60% conversion to the product. The mixture was filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the two isomeric ethyl ester products as white solids (major isomer, 26 mg, 54%)/(minor isomer, 6 mg, 13%). The major isomer was dissolved in MeCN (1 mL) and a solution of LiOH monohydrate (22.8 mg, 544.0 μmol , 15.0 eq.) in H_2O (1.1 mL) was added and the mixture was stirred at RT for 22 h. UPLC-MS analysis showed complete conversion to the desired product. The mixture was acidified with conc. HCl, filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (15 mg, **63%**). NOE analysis confirmed the correct structure of the product.

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 12.15 (s, 1H), 8.67 (t, J = 5.6 Hz, 1H), 8.30 (t, J = 5.8 Hz, 1H), 8.14 (t, J = 6.0 Hz, 1H), 8.02 – 7.97 (m, 1H), 7.96 – 7.92 (m, 1H), 7.91 – 7.86 (m, 4H), 7.60 (d, J = 1.6 Hz, 1H), 7.54 – 7.50 (m, 2H), 7.34 – 7.27 (m, 2H), 7.08 – 6.98 (m, 2H), 5.96 (d, J = 1.4 Hz, 1H), 3.45 – 3.40 (m, 2H), 3.34 (q, J = 6.6 Hz, 2H), 3.15 (dt, J = 7.9, 6.0 Hz, 2H), 2.78 (dt, J = 8.4, 6.1 Hz, 2H), 2.31 (d, J = 1.2 Hz, 3H), 1.79 (p, J = 6.8 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, $\text{DMSO}-d_6$) δ /ppm: 168.36, 167.51, 165.23, 150.72, 147.47, 139.95, 138.82, 138.51, 135.96, 134.99, 133.95, 133.22, 132.55, 132.08, 129.18, 128.35, 128.34, 128.24, 127.55, 127.00, 126.79, 126.01, 125.87, 124.39, 120.66, 48.32, 41.28, 38.56, 36.53, 28.09, 13.03.

HRMS (ESI): $C_{31}H_{31}N_7NaO_8S^+$ *calcd*: 684.1847, *found*: 684.1859.

9.29 Synthesis of NP20



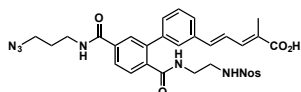
The isolated minor isomer from NP19 synthesis (5.0 mg, 7.3 μ mol, 1.0 eq.) was dissolved in MeCN (200 μ L) and a solution of LiOH monohydrate (4.6 mg, 109.0 μ mol, 15 eq.) in H₂O (217 μ L) was added. The solution was stirred at RT for 22 h after which UPLC-MS analysis showed full conversion. The solution was acidified with HCl (2 M) and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (3 mg, **63%**). NOE analysis confirmed the correct structure of the product.

1H NMR (500 MHz, DMSO- d_6) δ /ppm: 12.15 (s, 1H), 8.67 (t, J = 5.4 Hz, 1H), 8.33 – 8.25 (m, 2H), 8.13 (dt, J = 9.9, 5.8 Hz, 1H), 8.03 – 7.97 (m, 1H), 7.93 (dd, J = 6.2, 3.2 Hz, 1H), 7.90 – 7.86 (m, 4H), 7.56 – 7.48 (m, 2H), 7.44 (dt, J = 7.6, 1.5 Hz, 1H), 7.35 – 7.28 (m, 2H), 7.02 (d, J = 16.1 Hz, 1H), 5.76 (s, 1H), 3.44 – 3.39 (m, 3H), 3.36 – 3.33 (m, 2H), 3.17 – 3.12 (m, 2H), 2.74 (dt, J = 8.3, 6.1 Hz, 2H), 2.08 (d, J = 1.3 Hz, 3H), 1.79 (td, J = 6.7, 1.3 Hz, 2H).

^{13}C NMR (500 MHz, HMBC/HMQC, DMSO- d_6) δ /ppm: 168.28, 166.77, 165.23, 149.53, 147.30, 140.01, 138.82, 138.56, 136.23, 135.02, 134.40, 133.92, 132.52, 129.16, 128.46, 128.43, 128.11, 127.50, 127.34, 127.10, 126.00, 125.69, 125.54, 124.34, 118.65, 48.28, 41.22, 38.50, 36.48, 28.04, 20.18.

HRMS (ESI): $C_{31}H_{31}N_7NaO_8S^+$ *calcd*: 684.1847, *found*: 684.1857.

9.30 Synthesis of NP21



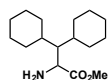
In a dry Schlenk tube 2-methyl-triethyl-4-phosphonocrotonate (44.5 mg, 135.0 μ mol, 1.3 eq.) was dissolved in THF (1 mL) and cooled to 0°C. n-BuLi (97.1 μ L, 155.3 μ mol, 1.5 eq. 1.6 M in hexanes) was added and the yellow solution was stirred at 0°C for 15 min. **XZ50** (60.0 mg, 104.0 μ mol, 1.0 eq.) was added and the yellow solution was stirred at 0°C for 15 min. The ice bath was removed and the solution was stirred at RT for 25 h after which UPLC-MS analysis showed about 66% conversion to the product. The solvent was removed by rotary evaporation and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the ethyl ester product as a white solid (41 mg, 57%). The material was dissolved in MeCN (1.5 mL) and a solution of LiOH monohydrate (24.9 mg, 594.0 μ mol, 10.0 eq.) in H₂O (743 μ L) was added and the mixture was stirred at RT for 23 h. UPLC-MS analysis showed complete conversion to the desired product. The mixture was acidified with conc. HCl, filtered and purified by reversed-phase preparative HPLC (Method A). Product-containing fractions were combined and lyophilized to yield the desired product as a white solid (30 mg, **76%**).

1H NMR (500 MHz, DMSO- d_6) δ /ppm: 8.67 (t, J = 5.7 Hz, 1H), 8.34 (t, J = 5.9 Hz, 1H), 8.14 (t, J = 6.0 Hz, 1H), 8.01 – 7.97 (m, 1H), 7.96 – 7.92 (m, 1H), 7.91 – 7.85 (m, 4H), 7.62 (s, 1H), 7.57 – 7.49 (m, 2H), 7.33 (t, J = 7.5 Hz, 1H), 7.30 (dt, J = 7.6, 1.6 Hz, 1H), 7.27 – 7.18 (m, 2H), 6.97 (d, J = 13.4 Hz, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.37 – 3.32 (m, 2H), 3.16 (dt, J = 7.9, 6.0 Hz, 2H), 2.79 (dt, J = 8.4, 6.1 Hz, 2H), 1.98 (d, J = 1.2 Hz, 3H), 1.79 (p, J = 6.8 Hz, 2H).

^{13}C NMR (126 MHz, DMSO) δ /ppm: 169.11, 168.68, 165.50, 147.70, 140.20, 139.17, 138.78, 138.28, 137.63, 136.38, 135.22, 134.10, 132.72, 132.57, 129.37, 128.64, 128.57, 128.43, 128.06, 127.71, 126.98, 126.36, 126.19, 124.64, 124.57, 48.57, 41.52, 38.85, 36.78, 28.33, 12.86.

HRMS (ESI): $C_{31}H_{31}N_7NaO_8S^+$ *calcd*: 684.1847, *found*: 684.1854.

9.31 Synthesis of AA008



Under an inert atmosphere *rac*-H- β , β -Dicyclohexyl-Ala-OH (100.0 mg, 395.0 μ mol, 1.0 eq.) was suspended in MeOH (3 mL) and cooled to 0°C. SOCl₂ (288 μ L, 4.0 mmol, 10.0 eq.) was slowly added whereby a clear solution formed. The solution was stirred in the ice bath for 10 min and was then warmed to RT. After 20 h another portion of SOCl₂ (288 μ L, 4.0 mmol, 10.0 eq.) was added and the solution continued stirring. After 72 h more SOCl₂ (144 μ L, 2.0 mmol, 5.0 eq.) was added. After total 7 d UPLC-MS analysis showed >98% conversion to the product. The volatiles were removed by rotary evaporation and the residue was dissolved in DCM (20 mL)

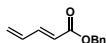
and washed with half-saturated NaHCO₃ solution (3 x 20 mL). The combined aqueous layers were extracted with DCM (2 x 20 mL) and the combined organic layers were washed with brine (20 mL), dried over Na₂SO₄ and concentrated *in vacuo* to yield the desired product as a yellowish oil (94 mg, **89%**).

¹H NMR (400 MHz, Methylene Chloride-*d*₂) δ/ppm: 3.66 (s, 3H), 3.61 (d, *J* = 2.6 Hz, 1H), 1.74 – 1.54 (m, 11H), 1.41 – 1.37 (m, 1H), 1.28 – 1.03 (m, 11H).

¹³C NMR (101 MHz, CD₂Cl₂) δ/ppm: 178.61, 54.59, 52.37, 52.14, 38.83, 36.88, 32.89, 32.43, 32.42, 30.61, 27.66, 27.65, 27.60, 27.51, 27.32, 27.20.

HRMS (ESI): C₁₆H₃₀NO₂⁺ *calcd*: 268.2271, *found*: 268.2272.

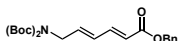
9.32 Synthesis of Benzyl (*E*)-penta-2,4-dienoate XZ53



Pentadienoic acid (200.0 mg, 2.0 mmol, 1.0 eq.) was suspended in DCM (3 mL) and benzylic alcohol (422.0 μL, 4.1 mmol, 2.0 eq.) was added. The mixture was cooled in an ice bath and EDC hydrochloride (782.0 mg, 4.1 mmol, 2.0 eq.) and DMAP (167.0 mg, 1.4 mmol, 0.7 eq.) were added. The mixture was warmed to RT and stirred for 5.5 h. HCl solution (1 M) was added to acidify the mixture and the phases were separated. The aqueous phase was extracted with DCM (2 x 8 mL) and the combined organic layers were washed with saturated NaHCO₃ solution, dried over Na₂SO₄ and concentrated by rotary evaporation. The crude was purified by column chromatography (Silica, 50 g, cyclohexane:EtOAc 1:1) to yield the desired product as a colorless liquid (271 mg, **71%**). Analytical data was in agreement with reported data.^[17]

¹H NMR (400 MHz, CDCl₃) δ/ppm: 7.41 – 7.27 (m, 6H), 6.46 (dt, *J* = 17.1, 10.1 Hz, 1H), 5.96 (d, *J* = 15.6 Hz 1H), 5.62 (d, *J* = 17.1 Hz, 1H), 5.50 (d, *J* = 10.1 Hz, 1H), 5.20 (s, 2H).

9.33 Synthesis of Benzyl (2*E*, 4*E*)-6-(bis-(Boc)amino)hexa-2,4-dienoate XZ54



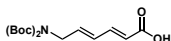
XZ53 (100.0 mg, 531.0 μmol, 1.0 eq.) and bis-Boc allylamine (342.0 mg, 1.3 mmol, 2.5 eq.) were dissolved in DCM (1.5 mL). A solution of the Hoveyda-Grubbs catalyst (23.3 mg, 37.2 μmol, 7.0 mol%) in DCM (1.2 mL) was added and the reaction mixture was stirred at 40°C for 16 h. The reaction mixture was concentrated by rotary evaporation and purified by preparative reversed phase HPLC (Method A, no TFA) to yield the desired product as a greenish solid (70 mg, **32%**).

¹H NMR (400 MHz, CD₃CN) δ/ppm: 7.45 – 7.62 (m, 6H), 6.33 – 6.625 (m, 1H), 6.23 – 6.14 (m, 1H), 5.97 (d, *J* = 15.4 Hz, 1H), 5.16 (s, 2H), 4.25 (d, *J* = 5.5 Hz, 2H), 1.46 (s, 18H).

¹³C NMR (101MHz, CD₃CN) δ/ppm: 167.2, 153.1, 145.0, 139.8, 137.6, 129.7, 129.5, 129.0, 129.0, 121.8, 83.2, 66.7, 48.3, 28.2.

HRMS (ESI): C₂₃H₃₁NNaO₆⁺ *calcd*: 440.2044, *found*: 440.2051.

9.34 Synthesis of (2*E*, 4*E*)-6-(bis-(Boc)amino)hexa-2,4-dienoic acid XZ55



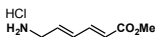
XZ54 (60.0 mg, 144.0 μmol, 1.0 eq.) was dissolved in H₂O (1.2 mL) and MeCN (1.2 mL) and LiOH monohydrate (90.5 mg, 2.2 mmol, 15 eq.) was added. The mixture was stirred at RT for 18 h. The mixture was purified by preparative reversed phase HPLC (Method A, no TFA) to yield the desired product as a brownish solid (32 mg, **68%**).

¹H NMR (500 MHz, DMSO-*d*₆) δ/ppm: 6.71 – 6.63 (m, 1H), 6.18 – 6.10 (m, 1H), 5.78 – 5.70 (m, 2H), 4.11 (d, *J* = 6.4 Hz, 2H), 1.43 (s, 18H).

¹³C NMR (126 MHz, DMSO-*d*₆) δ/ppm: 169.4, 151.8, 134.7, 134.4, 131.8, 130.1, 81.8, 47.4, 27.6.

HRMS (ESI): C₁₆H₂₅NNaO₆⁺ *calcd*: 350.1574, *found*: 350.1576.

9.35 Synthesis of AA076



XZ55 (32.0 mg, 97.7 μmol, 1.0 eq.) was suspended in MeOH (5.3 mL) and cooled in an ice bath. SOCl₂ (71.3 μL, 97.7 μmol, 10 eq.) was added and the mixture was stirred for 15 min at 0°C. The cooling bath was removed and the mixture was stirred at RT for 3 h.

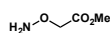
The volatiles were removed by rotary evaporation and the crude was recrystallized from MeOH/Et₂O to yield methyl the desired product as a brown solid (10 mg, **58%**).

¹H NMR (400 MHz, MeOD-*d*₄) δ/ppm: 7.31 (dd, *J* = 15.4, 10.9 Hz, 1H), 6.59 (dd, *J* = 15.4, 11.0 Hz, 1H), 6.19 (dt, *J* = 15.4, 6.4 Hz, 1H), 6.06 (d, *J* = 15.4 Hz, 1H), 3.75 (s, 3H), 3.69 (d, *J* = 6.4 Hz, 2H).

¹³C NMR (126 MHz, MeOD-*d*₄) δ/ppm: 169.5, 144.0, 134.5, 133.5, 124.2, 52.2, 41.8.

HRMS (ESI): C₇H₁₂NO₂⁺ *calcd*: 142.0863, *found*: 142.0863.

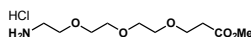
9.36 Synthesis of AA077



Aminoxyacetic acid (1.0 g, 11.0 mmol, 1.0 eq.) was suspended in MeOH (25 mL) and cooled to 0°C. SOCl₂ (4.0 mL, 54.9 mmol, 5.0 eq.) was slowly added and the solution was stirred at 0°C for 10 min. The cooling bath was removed and the solution was stirred at RT for 20 h. The solvent was removed by rotary evaporation and the residue was dissolved in half-saturated NaHCO₃ solution (25 mL). The mixture was extracted with EtOAc (3 x 25 mL) and the combined organic layers were washed with brine (25 mL), dried over Na₂SO₄ and concentrated. The crude material was purified by bulb-to-bulb distillation (100°C, 90 mbar). The desired product was isolated as colorless liquid (300 mg, **26%**). Analytical data was in agreement with reported data.^[18]

¹H NMR (400 MHz, Chloroform-*d*) δ/ppm: 4.48 (s, 2H), 3.80 (s, 3H).

9.37 Synthesis of AA078



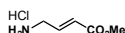
3-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]propanoic acid (118.0 mg, 533.0 μmol, 1.0 eq.) was dissolved in MeOH (3 mL) and cooled in an ice bath. SOCl₂ (389.0 μL, 5.3 mmol, 10.0 eq.) was dropwise added and the mixture was stirred at 0°C for 10 min. The ice bath was removed and the mixture was stirred at RT for 14 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed by rotary evaporation and the residue was dissolved in MeOH (5 mL), filtered and concentrated to yield the desired product as yellow oil (121 mg, **84%**).

¹H NMR (500 MHz, Methanol-*d*₄) δ/ppm: 3.75 (t, *J* = 6.1 Hz, 2H), 3.73 – 3.70 (m, 2H), 3.69 – 3.67 (m, 3H), 3.67 – 3.65 (m, 3H), 3.66 – 3.59 (m, 5H), 3.15 – 3.11 (m, 2H), 2.60 (t, *J* = 6.1 Hz, 2H).

¹³C NMR (126 MHz, MeOD) δ/ppm: 173.91, 71.52, 71.34, 71.20, 71.18, 67.83, 67.60, 52.18, 40.68, 35.62.

HRMS (ESI): C₁₀H₂₂NO₅⁺ *calcd*: 236.1492, *found*: 236.1494.

9.38 Synthesis of AA087



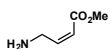
NaH in mineral oil (38.5 mg, 1.0 mmol, 1.6 eq. 60%) was suspended in THF (3 mL) and the mixture was cooled to 0°C. Methyl 2-diethoxyphosphorylacetate (173.0 μL, 942.0 μmol, 1.5 eq.) was added and the solution was stirred at 0°C for 15 min. 2-(*N*-Boc)acetaldehyde (100.0 mg, 628.0 μmol, 1.0 eq.) was added and the mixture was stirred at this temperature for 15 min. The cooling bath was removed and the solution was stirred at RT for 3.5 h after which TLC showed full conversion of the starting material. The reaction was quenched by the addition of EtOH (3 mL). The solvent was removed by rotary evaporation and the crude was purified by flash column chromatography (Silica, 37 g, 4:1 cyclohexane:EtOAc, *R*_f = 0.28, KMnO₄). Product-containing fractions were combined and concentrated to yield the Boc protected intermediate as a colorless oil (77 mg, 57%). The material was dissolved in DCM (2 mL) and cooled in an ice bath. HCl in dioxane (895.0 μL, 3.6 mmol, 10.0 eq. 4 M) was slowly added and the solution was stirred at 0°C for 10 min. The cooling bath was removed and the mixture was stirred at RT for 16 h. The volatiles were removed by rotary evaporation and the residue was co-evaporated with DCM (5 mL) to yield the desired product as beige solid (47 mg, **86%**).

¹H NMR (500 MHz, DMSO-*d*₆) δ/ppm: 8.28 (brs, 3H), 6.87 (dt, *J* = 16.0, 5.6 Hz, 1H), 6.18 (dt, *J* = 16.0, 1.8 Hz, 1H), 3.71 (s, 3H), 3.70 – 3.66 (m, 2H).

¹³C NMR (126 MHz, DMSO) δ/ppm: 165.36, 140.61, 123.35, 51.71, 40.11.

HRMS (ESI): C₅H₁₀NO₂⁺ *calcd*: 116.0706, *found*: 116.0707.

9.39 Synthesis of AA088



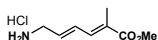
18-crown-6 ether (216.0 mg, 817.0 μmol , 1.3 eq.) was dissolved in THF (3 mL) and KHMDS (150.0 mg, 754.0 μmol , 1.2 eq.) was added. The yellow solution was cooled to -78°C and methyl 2-[bis(2,2,2-trifluoroethoxy)phosphoryl]acetate (147.0 μL , 691.0 μmol , 1.1 eq.) was added and the solution was stirred at 0°C for 5 min. 2-(*N*-Boc)acetaldehyde (100.0 mg, 628.0 μmol , 1.0 eq.) was added and the solution was stirred at -78°C for 1 h. The cooling bath was removed and the solution was stirred at RT for 1 h after which TLC showed full conversion of the starting material. The reaction was quenched by the addition of EtOH (3 mL). The solvent was removed by rotary evaporation and the crude was purified by flash column chromatography (Silica, 37 g, 5:1 cyclohexane:EtOAc, R_f = 0.23, KMnO_4). Product-containing fractions were combined and concentrated to yield the Boc protected intermediate as a beige solid (60 mg, 44%). The material was dissolved in DCM (2 mL) and cooled in an ice bath. HCl in dioxane (696.0 μL , 2.8 mmol, 10.0 eq. 4 M) was slowly added and the solution was stirred at 0°C for 10 min. The cooling bath was removed and the mixture was stirred at RT for 15 h. The volatiles were removed by rotary evaporation and the residue was co-evaporated with DCM (5 mL) to yield the desired product as beige solid (41 mg, **96%**).

^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ /ppm: 8.21 (brs, 3H), 6.37 (dt, J = 11.7, 5.9 Hz, 1H), 6.08 (dt, J = 11.5, 2.2 Hz, 1H), 4.00 (s, 2H), 3.69 (s, 3H).

^{13}C NMR (126 MHz, DMSO) δ 165.51, 141.74, 121.89, 51.50, 37.58.

HRMS (ESI): $\text{C}_5\text{H}_{10}\text{NO}_2^+$ *calcd*: 116.0706, *found*: 116.0708.

9.40 Synthesis of AA088



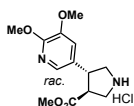
Under an inert atmosphere in a Schlenk tube methyl (*E*)-3-bromo-2-methylprop-2-enoate (250.0 mg, 1.4 mmol, 1.0 eq.), bis-(Boc)-allylamine (719.0 mg, 2.8 mmol, 2.0 eq.), $\text{Pd}(\text{OAc})_2$ (9.4 mg, 41.9 μmol , 3 mol%), tris(*o*-tolyl)phosphine (25.5 mg, 83.8 μmol , 6 mol%) and triethylamine (389.0 μL , 2.8 mmol, 2.0 eq.) were mixed and heated to 100°C and stirred at this temperature for 19 h after which UPLC-MS analysis showed complete conversion. The black mixture was quenched with NaOH (10%, 10 mL) and extracted with MtBE (3 x 10 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4 and concentrated by rotary evaporation. The crude material was purified by flash column chromatography (Silica, 95g, cyclohexane:EtOAc 20:1 \rightarrow 10:1, R_f = 0.20, UV/ KMnO_4) to yield the desired product as yellow oil (341 mg, 69%). The material (287.0 mg, 807 μmol , 1.0 eq.) was dissolved in DCM (4 mL) and cooled in an ice bath. HCl in dioxane (4.0 mL, 16.1 mmol, 20.0 eq 4 M in dioxane) was added and the solution was stirred at 0°C for 15 min. The mixture was warmed to RT and stirred for 19 h after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed by rotary evaporation and the residue was dissolved in MeOH (3 mL). The mixture was filtered by syringe filter, cooled in an ice bath to 0°C and Et_2O (5 x 3 mL) was added portionwise. The formed suspension was continued stirring at 0°C for 30 min and the precipitate was filtered off, washed with cold Et_2O (10 mL), dried *in vacuo* to yield the desired product as a white solid (100 mg, **65%**).

^1H NMR (400 MHz, Methanol- d_4) δ /ppm: 7.19 (d, J = 11.3 Hz, 1H), 6.82 (ddd, J = 15.2, 11.3, 1.7 Hz, 1H), 6.11 (dt, J = 15.2, 6.7 Hz, 1H), 3.76 (d, J = 1.7 Hz, 3H), 3.71 (d, J = 6.7 Hz, 2H), 1.99 (s, 3H).

^{13}C NMR (101 MHz, MeOD) δ /ppm: 169.88, 137.43, 132.10, 131.96, 130.75, 52.52, 42.03, 12.93.

HRMS (ESI): $\text{C}_8\text{H}_{14}\text{NO}_2^+$ *calcd*: 156.1019, *found*: 156.1018.

9.41 Synthesis of AA106



(*Trans-racemic*) 1-*tert*-butyl-3-methyl-4-(5,6-dimethoxypyridin-3-yl)pyrrolidine-1,3-dicarboxylate (250 mg, 682 μmol , 1.0 eq.) and triethylsilane (1.1 mL, 6.8 mmol, 10.0 eq.) were dissolved in DCM (3 mL) and cooled in an ice bath. HCl in dioxane (1.7 mL, 6.8 mmol, 10.0 eq. 4 M in dioxane) was added dropwise over 5 min and the solution was stirred in the ice bath for 10 min. The cooling bath was removed and the solution was stirred at RT for 50 min after which UPLC-MS analysis showed full conversion to the desired product. The volatiles were removed by rotary evaporation and the crude was purified by reversed phase column chromatography on the ISOLERA (RP- Silica, 100 g, $\text{H}_2\text{O}:\text{MeCN}$, UV). Product-containing fractions were combined and lyophilized to yield the desired product as a white, hygroscopic solid (38 mg, **16%**).

^1H NMR (500 MHz, Deuterium Oxide) δ /ppm: 7.65 (d, J = 1.9 Hz, 1H), 7.37 (d, J = 2.1 Hz, 1H), 3.97 (s, 3H), 3.91 (s, 3H), 3.87 (ddd, J = 11.7, 8.5, 5.0 Hz, 2H), 3.80 – 3.73 (m, 2H), 3.69 (s, 3H), 3.61 – 3.55 (m, 1H), 3.46 (t, J = 11.4 Hz, 1H).

^{13}C NMR (126 MHz, D_2O) δ /ppm: 172.81, 153.96, 144.19, 135.21, 126.19, 117.38, 55.71, 53.91, 52.95, 50.82, 48.60, 47.05, 44.36.

HRMS (ESI): $C_{13}H_{19}N_2O_4^+$ *calcd*: 267.1339, *found*: 267.1338.

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